THE INFLUENCE OF DEVELOPMENTAL TEMPERATURE ON SPERM FORM AND FUNCTION IN *CALLOSOBRUCHUS MACULATUS*

Ramakrishnan Vasudeva

A thesis submitted in partial fulfilment of the requirements of the University of Lincoln for the degree of Doctor of Philosophy

MARCH 2014

I dedicate this work to my Mother, Maya, Paul & Charles

Acknowledgements

First and foremost, I sincerely thank both my supervisors Dr. Paul Eady and Dr. Charles Deeming for their support and encouragement during the past three years, without which I could never have been able to write this. In 2006, I came to Lincoln to embark on a research project to study sexual conflict coevolution in the Bruchid beetle *Callosobruchus maculatus*. Thus, with the completion of my first research project the scene was set for a PhD in January 2010. I am forever in admiration of my insect populations as they provided me with a means for the past six years to learn about their Biology and apply it to study the processes of evolution. In that spirit I am truly indebted to my beetles (and to all those hundreds of beetles which I dissected).

Paul and I have worked together for over six years now and throughout he has been an ace mentor and a great inspiration. He played a significant role in welding together the multifarious branches of knowledge. He also went to great troubles in making me understand the importance of maintaining consistency in all my work (lab assays, data management, statistical analysis and writing). Regular meetings turned out to be the lynchpin of my understandings and the primary source to clarify doubts and avail research ideas. He taught me to maintain a balance and focus on the facts: a way to replace my inconsistencies. I shall remain indebted to him forever for being a superb guide and I am fortunate to be able to call Paul a colleague and friend.

Special thanks go to Dr. Charles Deeming for guiding me during the past three years. I shall remain forever grateful to him for providing support and insightful discussions on thermal switch experiments, ectothermic development and scientific writing. Similarly, I thank Dr. Daniel Pincheira-

Donoso; his advice on various aspects of the PhD were extremely helpful. He made valuable suggestions on acquiring key references in thermal biology and evolutionary biology. Thanks are due to Prof. Geoff Parker for being patient with my requests for papers on several occasions. I also thank the departmental regulars at 'Cake Fridays' and lunchtime conversations; they brightened me up.

I will remain grateful to Dr. William Hayes for being an excellent advisor on all aspects of scientific investigation. Throughout my time doctoral research, Bill has offered wonderful tutelage and sharing a work space with him was indeed great fun. I will miss him and our conversations over coffee and lunch breaks. In the same breath, I thank Mrs. Amanda Mylett for her help which made life easier.

I thank my family and friends who have unconditionally supported me to pursue my dreams. In this regard, my sincere thanks are due to my mother, Mrs. Gomathy Ramaswamy, Kenny & Alison Bell, who I think were my parents in England. Ken and Ali always looked after me like one of their own. I am forever grateful to my friends Dr. Kandasamy Bharathi and Dr. Needhirajan for their support. Thanks also go to Miss. Jacqueline Mayer who has been extremely supportive and many-a-time the driving force. Thanks are due to my Uncle Mr. R. Perumalswamy, whose unhindered financial support helped me focus just on my PhD. I also thank Gopal and Mahua for being there and supporting me always. Thanks are due to my wife Maya Kumar, her parents (Mr. K. S. Kumar and Mrs. Radha Kumar) and sister (Miss. Nidhi Kumar) for their support (in a myriad ways) during the writing up stages, which let me complete what I had started. Maya has been an excellent support in every way. I admire her steadfastness and those uplifting moments

whenever I lost focus. Now, I can spend more time with her and we look forward to our future together.

In conclusion, I gratefully acknowledge the funding I received towards my PhD from the University of Lincoln. I remain forever grateful to numerous researchers worldwide for their valuable contributions and my thanks are due to the Miss. Denise Bateman and the Library acquisitions team at the University of Lincoln for their support.

Abstract

This thesis examines the effects of the thermal rearing environment on larval development and its influence on the expression of primary reproductive traits in *Callosobruchus maculatus*.

Chapter 1 provided basic outline to the thesis, starting with a general introduction to sexual selection and specifically its role in the evolution of genitalia and primary reproductive traits. These traits appear to exhibit a level of phenotypic plasticity and thus the opening chapter introduces this concept and its role in evolution and diversification. Phenotypic plasticity in the primary reproductive traits of insects and the evolutionary consequences of this plasticity are discussed.

Chapter 2 examined the effect of larval rearing temperature on larval development and offspring size. Larvae go through 4 larval instars prior to pupation. Unsurprisingly larvae reared at 17°C took approximately four times longer to develop than those larvae reared at 33°C but those beetles that completed their development at the coolest temperature were the largest, in agreement with Bergmann's rule. This chapter paved way for the experiments carried out in subsequent chapters.

Chapter 3 investigated the effect of developmental temperature on the expression of primary reproductive traits (e.g. sperm length and egg size) and life-history traits (e.g. adult longevity and fecundity). Sperm length exhibited phenotypic plasticity, being shortest when larvae developed at the temperature

extremes (17°C & 33°C). Beetles raised at 17°C inseminated the fewest sperm despite the fact that these males had the largest absolute testes size. Developmental temperature affected female fecundity; females that underwent larval development at 17°C laid the fewest eggs. These females along with those reared at 33°C also laid the largest eggs. In general egg size tended to increase with decreasing developmental temperature (in agreement with Bergmann's rule) although sperm size gives a more mixed response being largest at an intermediate temperature. This is in agreement with the effect of developmental temperature on sperm size in dung flies, but in guppies and a species of snail sperm size increased with decreasing temperature.

Chapter 4 used thermal switch experiments to identify when, during larval development, the expression of sperm length was sensitive to temperature. Thermal switching involves an abrupt shift in the thermal environment experienced by the developing larvae, such that development starts at one environment and is completed at either a higher or lower thermal rearing environment. There appeared to be two thermal sensitive periods (TSP) for the expression of sperm length: one during the very stages of ontogeny and one around the time of instar III to instar IV. This is the first demonstration of a TSP in relation to the phenotypic expression of the sperm. The findings of this chapter are discussed in relation to previous shift-once or shift-twice experiments on reptilian embryos and a handful of insects.

Chapter 5 studied the consequences of thermal environment during larval development and as adults on copula duration. Both larval rearing temperature

and post-eclosion temperature affected copula duration; males reared at the lowest temperature taking longer to complete copulation. This is the first demonstration that larval rearing temperature affects copulatory behaviour. It is argued that because thermal heterogeneity is likely to be common in nature, this under-examined component of male behaviour could account for much variation in copulatory behaviour in nature.

Chapter 6 examined the effect of larval rearing temperature on the outcome of sperm competition. Males reared at 17°C were less successful at sperm defence and sperm offence, whilst males reared at 33°C were less successful at sperm offence in comparison to males reared at 27°C. This is the first study to report an effect of developmental temperature on the outcome of postcopulatory sexual selection. The results are discussed in light of the physiological consequences of developmental temperature on male ejaculatory characteristics.

Chapter 7 discusses the possible mechanisms and consequences of phenotypic plasticity in reproductive traits as a result of experiencing heterogeneous environments during development, and how these processes might interact with postcopulatory sexual selection. Further work on understanding the role of phenotypic plasticity in the evolution of primary reproductive traits is required.

Contents

1. General Introduction

1.1 Sexual selection

Primary reproductive characters are those traits that relate to the reproductive organs and gametes of a species (Eberhard, 1985; Ghiselin, 2010). By contrast, secondary sexual characters are those traits that represent differences between the sexes other than those relating to the reproductive system, such as the antlers, horns tusks and sex combs of males or plumage/pelage differences (Andersson, 1994; Snook *et al.*, 2013). Darwin was the first to attempt to define primary reproductive traits, as those traits directly involved in sexual reproduction (e.g. sperm and eggs) (Ghiselin, 2010).

Primary reproductive traits are often useful entities in taxonomy, being used to distinguish between closely related species (Eberhard, 1985; Baccetti, 1986; Guidi & Rebecchi, 1996). This observation suggests that primary reproductive traits have been subjected to strong directional selection, resulting in their rapid and divergent evolution. However, what selection pressures drive this evolution are still poorly understood (Pitnick *et al.*, 2009), although the most popular and contemporary theory for their evolution is that of sexual selection (Eberhard, 1985; 1996; Hosken & Stockley, 2004; Leonard & Córdoba-Aguilar, 2010).

Prior to Parker's seminal work (Parker, 1970a), sexual selection was viewed as a precopulatory battle between males for access to females and/or female mate choice (Endler, 1980; Eberhard, 1985; Parker, 1992; Andersson, 1982, 1994; Eberhard 1996; Parker, 2006)**.** Male-male competition in some instances for example is viewed as a battle of horns or antlers (male fighting

ability) over access to reproductive females (Kodric-Brown & Brown, 1984; Birkhead & Møller, 1998). In other cases male-male competition extends beyond the aforementioned scenario to complex communication signals (via songs and odours) for attracting a mate, whereby a competitive male may yield direct and/or indirect fitness benefits on the female (Hebets & Papaj, 2005). For example, in the European red deer (*Cervus elaphus*), reproductive combats for mates take place between rival males, wherein secondary sexual characteristics (e.g. male antlers) are used to settle disputes over access to reproductive females. Antler size tends to correlate with social status/dominance (and nutritional condition) and antlers are often used during male threat displays. These large, elaborate structures are related to the fighting ability of the male, which directly increases the probability of mating (Clutton-Brock *et al.*, 1982; Kodric-Brown & Brown, 1984; Andersson, 1994).

In contrast to male-male competition, female choice involves female preferences for male traits that may or may not correlate with direct phenotypic benefits to the female (Andersson, 1994; Andersson & Simmons, 2006). For example, in the guppy (*Poecilia reticulata*), females actively seek brighter coloured males to mate with despite the fact that nuptial colouration tends to elevate the risk of being predated (i.e. being more conspicuous to predators). Such female preferences represent a central theme in behavioural ecology. Why in many species do females take such care in choosing a mate, when all the male appears to provide is sperm? Several mechanisms have been proposed to account for the evolution of female preferences. These include, i) Direct phenotypic benefits, ii) Sensory bias, iii) Fisherian sexy sons, iv) Handicap mechanisms (good genes) and v) Genetic compatibility (Andersson

& Simmons, 2006). These five mechanisms can occur in succession or be mutually compatible with each other, rendering the evolution of mate choice a multiple-causation problem (Andersson, 1994; Andersson & Simmons, 2006). Here I briefly describe each mechanism.

Direct phenotypic benefits associated with female mate preferences require the male ornament to reflect the male's ability to provide material advantages from which the female gains. For example, in the sand goby (*Pomatoschistus minutus*), females preferred to mate with males that had eggs in their nests as this was indicative of males being successful parents through greater investment in parental care (Forsgren *et al.*, 1996). Alternatively, female choice may evolve to reduce the direct costs imposed by males on females (Holland & Rice, 1998; Arnqvist & Rowe, 2005). For example, Rice & Holland (1997) have suggested that mating may be costly to females due to energy loss, and/or injury via genitalic damage; (Crudgington & Siva-Jothy, 2000) or harmful seminal products; (Rice, 1996) and/or the manipulation of female reproductive behaviour and physiology. Thus, Holland & Rice (1998) argue that females evolve resistance to male courtship displays that function to persuade females to mate with them. This obviously has its root in sexual conflict (see below and Arnqvist & Rowe, 2005).

Under the sensory bias model of female choice, females have pre-existing preferences for particular male traits. For example, female zebra finches (*Poephila guttata*) prefer male zebra finches that have white feathers glued to their heads (similar results have been found for plastic leg bands) (Burley *et al.*, 1982). Why they prefer such males is unknown, although it could be due to some naturally selected preference in females to find and

respond to the sight of white feathers; zebra finches tend to line their nests with white feathers (Burley *et al.*, 1982).

In Fisherian sexy sons, the mechanism of female mate choice takes shape by self-reinforcing coevolution. Certain male traits seem to be favoured by the female (maybe via pre-existing sensory bias). Those alleles associated with the male's appearance invariably get selected but in a self-reinforcing way such that females that buck the trend are selected against, because they will tend to produce sons that are less attractive to the majority of the female population. Here, the female preferences bring no direct benefits in terms of offspring survival, rather females benefit by having sexy sons (Andersson, 1994; Arnqvist & Rowe, 2005).

By contrast, the handicap mechanism proposes that the male's trait reveal his underlying genetic quality. Thus in choosing an ornate male, the female is effectively choosing 'good survival genes' (Andersson, 1994). For example, Petrie (1994) found that the offspring of female peacocks (*Pavo cristatus*) that had been paired with the most ornate males had enhanced growth and survival in comparison to those paired with less ornate males.

Under the genetic compatibility model, females mate with more than one male, as males naturally vary in their genetic compatibility with females (Tregenza & Wedell, 2000). This could involve both additive and non-additive genetic benefits in choosing to mate with a more compatible male that complements their genetic make-up (Andersson, 1994; Tregenza & Wedell, 2000). For example, under the genetic compatibility model females are believed to exploit post-copulatory mechanisms to minimize the risk of fertilization by genetically incompatible sperm (Zeh & Zeh, 1996). Zeh & Zeh

(1996) believe a number of processes (e.g. transposable elements, segregation distorters) modify maternal and paternal haplotypes in ways that render them incompatible within the developing embryo. Thus females mate with multiple males to hedge against genetic incompatibility. In support of this Zeh & Zeh (1997) found that females of the harlequin beetle- riding pseudoscorpion (*Cordylochernes scorpioides*), that were restricted to mating with a single male, experienced a higher rate of embryo failure than females mated to more than one male (see also Tregenza & Wedell, 2000).

Currently, there is no clear consensus regarding the mechanisms by which female preferences evolve, rather it appears female preferences are subject to a combination of selection pressures as described above (Andersson & Simmons, 2006).

1.2 Postcopulatory sexual selection

The recognition that females mate with multiple males means that sexual selection extends beyond copulation, where it takes the form of sperm competition, cryptic female choice and sexual conflict (Eberhard, 1996; Birkhead & Møller, 1998; Simmons, 2001; Birkhead & Pizzari, 2002; Arnqvist & Rowe, 2005). Postcopulatory sexual selection resulting from the promiscuity of the sexes has been described as a potent engine driving the evolution of molecules, inter-sexual specializations and population divergence (Birkhead & Pizzari, 2002). Male adaptations to sperm competition include prolonged copulation, multiple copulation, the production and insemination of large numbers of sperm (via larger testes), sperm removal (both direct and indirect), the insemination of seminal products that alter female reproductive

parameters and the restriction of female mating opportunities by mate guarding and/or copulatory plugs (Birkhead & Møller, 1998; Simmons, 2001; Shine *et al.*, 2008).

Sperm competition theory predicts that an increase in the risk of sperm competition should result in the production and insemination of greater numbers of sperm (Parker, 1970a; Parker *et al.*, 1992). This has been demonstrated numerous times across taxa; when sperm competition risk is high, males tend to have larger testes (Cook & Gage, 1995; Stockley *et al.*, 1997; Birkhead & Møller, 1998; Simmons & Siva-Jothy, 1998; Simmons, 2001; Birkhead & Pizzari, 2002). In addition, because ejaculates are costly to produce, selection will favour males that strategically tailor their ejaculate to either the risk of sperm competition and/or female reproductive status (Gage, 1991; Martin & Hosken, 2002; Uhía & Rivera, 2005).

Cryptic female choice (CFC) can be defined as female processes or structures that bias the utilization of sperm from different males at fertilization. These mechanisms are relatively less well studied than sperm competition (Eberhard, 1996; Birkhead, 1998; Birkhead & Pizzari, 2002), partly because, until recently, CFC was not considered a powerful agent of sexual selection (Thornhill, 1983, 1984; Eberhard, 1996) and, partly because it was difficult to study the interactions between males and females within the female reproductive environment. The selective use of sperm at fertilization by females can take the form of premature interruption of copulation, biasing the storage of sperm in favour of particular males, biasing the transport of stored sperm to the site of fertilization, biasing male (sperm) fertilization success at the surface of the ova, or denying male genitalic access necessary

for sperm transfer (Eberhard, 1996, Pizzari, 2004). CFC can result in directional or non-directional selection (Birkhead & Pizzari, 2002). Directional selection via cryptic female choice can result from females favouring the usage (bias) of certain types of sperm, resulting in the spread of the chosen alleles of those males within a population. For example, in the feral fowl, (*Gallus gallus domesticus*), females actively eject the sperm of lower ranked males. Females are thought to benefit from this behaviour by minimizing the costs of partner choice in this species (Pizzari & Birkhead, 2000). In contrast, non-directional CFC occurs when female sperm choice is associated with favouring certain compatible genotypes over others, irrespective of the male phenotype (see above & Tregenza & Wedell, 2000). This results in non-directional CFC. Non-directional CFC has been shown in self-fertilizing organisms (hermaphrodites), where females are predicted to favour the sperm of males that are genotypically compatible, regardless of their phenotype. However, the exact mechanism by which the male genotype is assessed by females is unknown (Birkhead & Pizzari, 2002).

Sexual conflict, first recognized by Trivers (1972), results from the conflict of interest between the sexes over fitness (Parker, 1979, 2006). Trivers (1972), described the asymmetries between males and females with regard to parental investment, in which one sex tended to invest more than the other sex in offspring. This asymmetry in investment is thought to stem from female gametes being larger than male gametes (anisogamy) leading to distinct roles as a result of differing evolutionary interests (Arnqvist & Rowe, 2005). In essence female reproductive success tends to be limited by access to resources, whereas male reproductive success tends to be limited by access to female

ova. Hence, it is usually males that compete for females. In some cases, these differing reproductive interests can result in sexual conflict (Ghiselin, 2010). Sexual conflict occurs when females choose to reject certain conspecific males as a direct result of avoiding costs of mating or the fact that those males are less attractive (Gavrilets *et al.*, 2001; Froman *et al.*, 2002; Arnqvist & Rowe, 2005). For example, in *Drosophila*, Rice (1996) showed that by experimentally preventing females from coevolving with freely evolving males (via an experimental evolution protocol) male fitness increased at a cost to their female counterparts; males were able to readily induce remating in females that had previously mated, resulting in an elevation in male fitness and an incidental reduction in female fitness due to toxic male seminal proteins and sub-optimal female mating rates (Rice, 1996).

There is good evidence that postcopulatory sexual selection plays a definitive role in the rapid evolution of genitalia via male-male competition, cryptic female choice and/or sexual conflict (Eberhard, 1985, 1996, 2010; Hosken & Stockley, 2004; Stockley, 1997; Leonard & Córdoba-Aguilar, 2010). Variation in genital morphology and success at fertilization has been shown to be related in the fly *Dryomyza anilis* (Otronen, 1998) and the water strider (*Gerris lateralis*) (Arnqvist & Danielsson, 1999a). In *G. lateralis*, males with stout ventral sclerites and narrow inwardly curved dorsal sclerites had greater reproductive success during sperm competition. The outcome of sperm competition in the dung beetle (*Onthophagus taurus*), was also shown to be influenced by genital morphology with four out of the five genital components measured being associated with performance in sperm competition (House & Simmons, 2002). In a different species of water strider,

Aquarius remigis, bigger males with longer legs were more active and thus secured more matings by encountering greater number of females. Additionally, bigger males had wider foreleg femurs and longer genitalia, enabling them greater success at overcoming female resistance (and attaining more matings). In contrast, females favoured smaller males due to males being lighter, with less expensive 'shields' against the harassment of bigger males (Sih *et al.*, 2002).

Sperm morphology varies dramatically between species (Snook, 2005; Garcia-Gonzales & Simmons, 2007; Rugman-Jones & Eady, 2008; Rönn *et al.*, 2011; Gage, 2012) but shows remarkable consistency within species (Beatty, 1970; Gage & Cook, 1994; Radwan, 1996; Morrow & Gage, 2000; Kleven *et al.*, 2008). Across species the variation in sperm size is 2,000-fold (Gage, 2012), despite the fact that the function of sperm, to fertilize ova, is fundamentally similar. The general consensus is that postcopulatory sexual selection and/or sexual conflict has driven the evolution of interspecific variation in sperm morphology (Schärer *et al.*, 2011). However, the numerous comparative studies (see Pitnick *et al.*, 2009; Gage, 2012) that have examined sperm morphological diversity in relation to female mating patterns have found inconsistent results. For example, in birds Immler & Birkhead (2007) found a positive association between midpiece length and flagellum length and relative testes size in the Fringillidae, but a negative association between the same traits in the Sylvidae. In fish, Stockley *et al.*, (1997) found a negative association between sperm length and intensity of sperm competition whilst Gage (1994) and Morrow & Gage (2000) found a positive relationship in butterfly species and moth species respectively. A number of studies have

found no relationship between sperm competition intensity and sperm length; Gage & Freckleton (2003); Minder *et al.*, (2005); Rugman-Jones & Eady, (2008); Fitzpatrick & Baer, (2011).

A more consistent pattern to emerge from comparative studies of sperm morphological diversity is that of correlated evolution with aspects of reproductive morphology. For example, when flatworms (Genus: *Macrostomum*) follow a reciprocal mating system in which male and female mating partners simultaneously play both male and female roles and an individual must transfer an ejaculate in order to be able to receive an ejaculate, sperm design is complex (stiff lateral bristles). By contrast, when mating is via hypodermic insemination, sperm design is simple. This association between mating type and sperm structure is likely to be related to female reproductive tract morphology, as species that practice hypodermic insemination have relatively simple female reproductive tracts (Schärer *et al.*, 2011; see Michiels, 1998). Indeed, interspecific studies tend to reveal positive relationships between sperm size and dimensions of female reproductive tract (Pitnick *et al.*, 2009; Gage, 2012). For example, a positive relationship between sperm length and the length of the spermatheca has been reported in featherwing beetles (Coleoptera: Ptiliidae) (Dybas & Dybas, 1981) and the length of the seminal receptacle in *Drosophila* (Pitnick *et al.*, 1999). In stalk-eyed flies (Diopsidae), dung flies (Scathophagidae), moths (Lepidoptera) and bruchid beetles there is a positive relationship between sperm length and spermathecal duct length (Presgraves *et al.*, 1999; Morrow & Gage 2000; Minder *et al.*, 2005; Rugman-Jones & Eady, 2008). Sperm length and sperm storage tubule length are also positively associated in birds (Briskie &

Montgomerie, 1992). These studies suggest that the female reproductive tract environment exerts a strong selection pressure on male (sperm) trait evolution. Indeed, in *Drosophila melanogaster*, Miller & Pitnick (2002) found that selection for increasing length of the ventral receptacle, the female sperm storage organ, was associated with a co-evolutionary increase in sperm length. Gage (2012) has argued that diversification of the female postcopulatory sexual arena could provide (honest) information regarding male quality during sperm competition, akin to the information gathered by females regarding a male's condition during precopulatory mate choice (Gage, 2012).

Within species variation in sperm length has been shown to be positively related to male body size (a proxy for male condition) in the guppy (*Poecilia reticulata*) (Skinner & Watt, 2007) whilst in the dung beetle, (*Onthophagous taurus*), smaller males had longer sperm (Simmons & Kotiaho, 2002). In *Drosophila bifasciata*, there was no relationship between sperm size and male size (Kurokawa *et al.*, 1974). A reduction in food quality or quantity had no effect on sperm size in the Indian meal moth, (*Plodia interpunctella*) (Gage & Cook, 1994), whilst studies into the effects of alternative mating strategies on sperm size have produced inconsistent results (Pitnick *et al.*, 2009). Experimental evolution studies, in which postcopulatory sexual selection was removed through enforced monogamy, had no effect on sperm length either in the yellow dung fly (*Scathophaga stercoraria*) (Hosken *et al.*, 2001), *Drosophila* (Pitnick *et al.*, 2001; also see Gage & Morrow, 2003; Hosken & Ward, 2008) or *C. maculatus* (Gay *et al.*, 2009). Thus, within species there are few if any, consistent ecological or physiological factors that correlate with variation in sperm length. An interesting observation that has

largely been ignored by sperm biologists is that in *S. stercoraria*, sperm length varied in response to developmental temperature (Blanckenhorn & Hellriegel, 2002). Males reared at cool temperatures (12°C & 15°C) produced relatively small sperm. By contrast, Minoretti *et al.* (2013) showed that rearing the land snail (*Arianta arbustorum*) at high temperatures (20°C) resulted in males with relatively small sperm. Breckels & Neff (2013) showed that male guppies (*Poecilia reticulata*) exposed to high developmental temperatures (30°C) resulted in shorter sperm. In some respects these findings are unsurprising because temperature affects many (if not all) of life's processes. I now discuss the extensive literature on growth and development in response to the thermal environment.

1.3 Thermal biology, phenotypic plasticity and the temperature-size rule

Of all the variables that shape and regulate the processes of life, temperature is the most profound variable that comes to mind, being an important property of matter that affects all processes pertaining to life. Thermal biology roots itself within the discipline of ecological physiology (Bennett, 1987; Angilletta, 2009).

Ninety nine percent of all animal species are ectothermic (Pincheira-Donoso *et al.*, 2008) in which metabolic rate is largely driven by environmental temperature. In ectotherms, metabolic rate determines the rate of growth (increase in mass) and the rate of development (passing through life-stages) and ultimately body size, which impacts on all aspects of life, including reproduction, mortality, dispersal, population abundance and energy flow (Forster *et al.*, 2011a). A widespread pattern in biology is the

temperature-size rule which refers to the general observation that within species, animals attain a smaller size when reared at higher temperatures (Pincheira-Donoso *et al.*, 2008). This phenotypically plastic response has been described as the third universal ecological response to global warming (Daufresne *et al.*, 2009). The temperature-size rule has also been described as a life-history puzzle (Sevenster, 1995) because it might be predicted that at higher temperatures organisms should exploit the faster growth rates and simply delay maturation, so that as adults they take advantage of the increase in survival, fecundity and mating success associated with larger body size (Kingsolver & Huey, 2008).

A simple explanation as to why size increases at low temperature is still lacking (Angilletta & Dunham, 2003; Walters & Hassall, 2006; Angilletta, 2009; Pincheira-Donoso, 2010), although the general consensus is that as temperature increases development rate increases more quickly than growth rate, such that an organism attains a smaller size at adulthood at higher temperatures (Forster *et al.*, 2011a). However, this simply leads to the next question: why should development rate increase at a greater rate than growth rate? One proposal is that growth depends primarily on protein synthesis whilst development depends on DNA replication. Protein synthesis is limited by the diffusion of large molecules into the cytoplasm, whilst DNA synthesis is limited by enzymatic processes, and thus it has been argued that development is more temperature sensitive than growth (van der Have & de Jong, 1996).

The temperature size rule is also apparent in altitudinal and latitudinal size clines with animals at higher altitudes and latitudes tending to be larger

(Walters & Hassall, 2006; Pincheira-Donoso, 2010). Although the trend of geographic clines in body size was originally described for endothermic organisms (Bergmann, 1847), later investigations extended this observation to ectotherms. The temperature-size rule, also used interchangeably with Bergmann's rule, was originally thought to be adaptive on the basis that an increase in body size at low temperatures reduces heat loss by the animal presenting a smaller surface area to volume ratio (Blanckenhorn & Hellriegel, 2002; Pincheira-Donoso *et al.*, 2008). However, this is unlikely to be the adaptive explanation for temperature-size rule in ectotherms.

The observed change in size (the phenotype) in relation to developmental temperature represents an example of phenotypic plasticity. Phenotypic plasticity refers to the property of a particular genotype being able to produce distinct phenotypes when exposed to variable environmental conditions (Gotthard & Nylin, 1995; Price *et al.*, 2003; Ghalambor *et al.*, 2007). The effects of temperature on life-history traits in poikilotherms are well known, although studies pertaining to the effect of temperature on metabolic pathways and physiological processes that result in developmental variation and the expression of phenotypic traits are fairly limited (Price *et al.*, 2003; DeWitt & Scheiner, 2004; Angilletta, 2009; Ghalambor *et al.*, 2007). A key concept in phenotypic plasticity is the reaction norm (Rodríguez, 2012). A reaction norm is a property of a genotype that describes how development maps the genotype into the phenotype as a function of the environment (Gotthard & Nylin, 1995; Nijhout *et al.*, 2010; Espinosa-Soto *et al.*, 2011). Plasticity represents a derivative of its reaction norm (Gotthard & Nylin, 1995; Price *et al.*, 2003; Angilletta, 2009; Ghalambor *et al.*, 2007) and for

phenotypic plasticity to evolve; there must be a change in the slope of the reaction norm between ancestral and derived species (Gotthard & Nylin, 1995). A reaction norm is equal to the slope of the function that describes the relationship between trait expression and developmental conditions (e.g. temperature, humidity or photoperiod). A trait that fails to display phenotypic plasticity has a reaction norm with a slope of zero and the intercept is the mean value of the trait (see Fig. 1) (Sultan, 2003; Angilletta, 2009). Plasticity tends to be recognized as either a graded or a discrete response. A graded response describes 'continuous phenotypic modulation' during an environmentally dependent developmental process, whereas discrete plasticity describes a situation in which a totally different phenotypic value (trait) arises in response to environmental change due to 'developmental conversion' e.g. polyphenism or polymorphism (see Nijhout, 2003a).

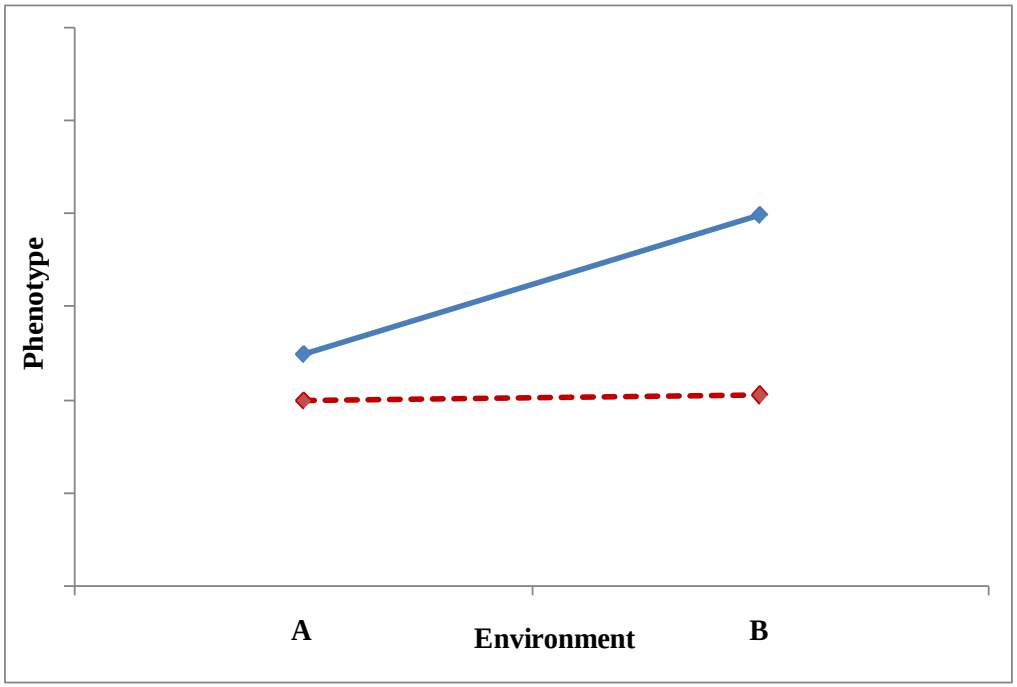


Fig. 1 The relationship between the phenotype and the environment. Blue line indicates a reaction norm. The slope of the line describes the relationship between the phenotypic expression and the two developmental environments

A and B. The red line represents a reaction norm with slope of 0 indicating no phenotypic plasticity in this trait.

When observed in the embryonic or juvenile stages of development (phenotypic plasticity) is often referred to as developmental plasticity (Stillwell & Fox, 2005; Gilbert, 2012). The evolution of plasticity can be quantified among traits, between populations or across taxa (Schmalhausen, 1949; Via *et al.*, 1995; Gibert *et al.*, 2000; Angilletta, 2009; Moczek, 2010; Pfennig *et al.*, 2010). All organisms exhibit phenotypic plasticity to some degree in their underlying biological processes (Nylin & Gotthard, 1998; West-Eberhard, 2005a; Gilbert, 2012).

Phenotypic plasticity can be mediated by biotic or abiotic environmental cues, e.g. parasitic load, predatory presence, ambient temperature or humidity (Andrade *et al.*, 2005) and recent studies in agriculture, ecology and developmental biology have shown phenotypic plasticity to be adaptive in numerous cases, although some authors question this (Via *et al.*, 1995; West-Eberhard, 2003; Moczek, 2010). Adaptive phenotypic plasticity in the expression of life-history traits, such as body size, longevity and reproductive strategy has been reported in a number of animal and plant studies (Via *et al.*, 1995; Sultan, 2003; Whitman & Agrawal, 2009; Moczek, 2010). For example, *Drosophila* larvae exposed to potentially lethal temperatures when feeding within sun-exposed fruit, express a biochemical defence mechanism via the induction of heat-shock proteins that stabilize the cells and protect the developing larvae from death (Neidhardt *et al.*, 1984). Grasshoppers, *Melanoplus femurrubrum*, grow bigger and stronger mouth parts when they experience low quality, fibrous food during early

developmental stages (Thompson, 1992). Similarly, in the suspension feeding barnacles (*Balanus glandula* Darwin), populations found in protected bays possess long thin cirri with long setae; whereas those found in wave-exposed shores have shorter, stouter cirri with shorter setae (Marchinko, 2003). The different forms of cirri are thought to be adaptive because in sheltered bays the long cirri enhance the surface area for prey capture where particle flux appears to be limited, whilst in wave-exposed environments short-cirri reduce damage arising from greater drag associated with more turbulent water flow (Marchinko & Palmer, 2003).

Several studies have reported phenotypic plasticity in response to developmental temperature. The environmental temperature experienced by developing tadpoles (*Rana cascadae*) affected subsequent leg length: faster growing tadpoles (higher temperature) attained longer legs at metamorphosis than slower growing tadpoles (Blouin & Brown, 2000). In the tropical butterfly (*Bicyclus anynana*), egg size and pupal mass increased at low temperature (20°C) compared to those individuals reared at 27°C (Fischer *et al.*, 2006). Gibert *et al.* (2001), compared the walking speeds of two populations of *D. melanogaster* derived from Congo and France. They showed that the walking speed of adult Congo files was faster than the France flies when the larvae were reared at high temperatures (25°C & 29°C), whereas the inverse was true when larvae were reared at 18°C, suggesting that reaction norms are population specific. Using genetic lines of *D. melanogaster* maintained at three different thermal environments (16.5°C over 10years, 25°C over 9years & 29°C over 4years), Gilchrist *et al.* (1997) showed that flies from the 16.5°C population had a greater locomotor speed than those

reared at 25°C and 29°C when assayed at 16.5°C. However, these 16.5°C flies also had greater walking speeds when assayed at 25°C and 29°C. Thus, the fitness of flies appears to be genetically coupled with tolerance to extreme high temperatures, with natural selection resulting in divergence in the thermal sensitivity of walking speeds.

Many studies of phenotypic plasticity are laboratory based, and relatively little is known about the effects of variation in environmental conditions on the expression of phenotypes in wild populaitons (Husby *et al.*, 2010). A long-term, study conducted to examine phenotypic plasticity in laying date and clutch size in two distinct populations of Great tit (*Parus major*) in the Netherlands and UK revealed that the average lay dates and clutch sizes for both populations were similar. Husby *et al.* (2010) were unable to detect a significant interaction between genotype and environment for laying date between the two populations, i.e. population level lay dates between the two populations were similar. However, laying date was shown to be significantly different between individuals within a population; lay date varied with environmental temperature between individuals (Husby *et al.*, 2010).

1.4 Adaptive plasticity or pathological effects?

Adaptive plasticity refers to developmental, physiological and reproductive adjustments that enhance function under specific environmental conditions and is distinct from pathological effects, such as resource limitation, on the expression of phenotypes (Gould & Vrba, 1982; Sultan, 1995, 2000; Angilletta *et al.*, 2004a,b; Gilbert, 2012). Plasticity itself is likely to be

favoured in populations with high genetic variability when, i) the population is exposed to frequent environmental fluctuations, ii) when such environmental fluctuations act as reliable cues to development, iii) different phenotypic traits are favoured at each of the variable environments and iv) when no individual phenotypic value possesses an advantage in all the environments (see Relyea, 2002; Ghalambor *et al.*, 2007). For many ectothermic organisms, temperature experienced during embryonic development plays a major role in determining growth and development rates (Gilbert, 2012). For example, as temperature decreases, developmental time and offspring size increase (Stillwell & Fox, 2005, 2007). The difficulty faced by scientists is to determine if the phenotype produced as a result of a plastic response is adaptive or not? Phenotypic plasticity is more often than not considered adaptive, although in some cases the environmentally induced traits appear non-adaptive (see de Jong, 2005; Ghalambor *et al.*, 2007). A recent study by Stillwell *et al.* (2008) attempted to test the adaptive nature of increasing body size at lower environmental temperatures. They created large and small beetles via artificial selection lines and then raised them at low and high temperatures. The large beetles had the greatest fitness at both temperatures, but the relative fitness advantage of large beetles did not differ between the two environments. Thus, being large at low temperatures does not appear to be adaptive in this case (but for examples of adaptive responses in other traits see (*Polygonum* spp.), Sultan, 2000; (*Daphnia magna*), Barata *et al.* 2001; ectotherms, Gilbert, 2001; collared fly catcher (*Ficedula albicollis*), Griffith-Simon & Sheldon, 2001; deer mice (*Peromyscus maniculatus sonoriensis*), Hammond *et al.* 2001; freshwater snail (*Potamopyrgus antipodarum*), Negovetic & Jokela, 2001; lizards

(*Microlophus delanonis*), Jordan & Snell, 2002; Wood frogs (*Rana sylvatica*), Relyea 2002, 2004.

In young tadpoles of the wood frog (*Rana sylvatica*), specific water-borne chemical signals derived from the presence of caged predators resulted in tadpoles hiding more, foraging less and producing longer tails with shorter bodies (and hence shorter guts) (Relyea & Auld, 2004). The longer tails enabled the tadpoles to swim faster and better avoid predation, suggesting the plasticity to be adaptive despite the fact that these tadpoles had less efficient digestion, grew slower and had a smaller body size (Relyea & Auld, 2004). The phenomenon of reduced gut length in the presence of predators has been reported in other larval amphibians and a wide variety of prey taxa suffer similar costs of slower growth, (e.g. *Daphnia*, fish, salamanders and snails; Gilbert, (2001); Relyea (2004); Relyea & Auld, (2004)). Of interest, intraspecific competition between wood frog tadpoles can also induce changes in gut length and foraging behaviour. Thus the stouter, shorter gutted individuals that resulted from the presence of predators could be a pathological response to a decreased feeding rate. This goes to show that adaptive plasticity is difficult to demonstrate (see Peacor & Werner, 2003; Relyea & Auld, 2004).

1.5 Ecological consequences of phenotypic plasticity

Recent studies have primarily focused on organisms' ability to display phenotypic plasticity under variable environmental conditions, whilst the ecological consequences of such plasticity have been seldom studied (Miner *et al.*, 2005). Phenotypic plasticity has been described as both a facilitator and an

inhibitor of diversification (Pigliucci, 1996; 2001; 2005; Pfennig *et al.*, 2010; Fitzpatrick, 2012). Traditionally, phenotypic plasticity was seen as an inhibitor of evolutionary change because if individuals can produce optimal phenotypes via plasticity in response to multiple environmental conditions, then genetic variation in the ability to survive and reproduce is hidden from selection. In effect, genetic alternatives are not required for attaining fitness optima (Pfennig *et al.*, 2010; Fitzpatrick, 2012) as different genotypes can produce the same phenotype via plasticity, and thus genetic differences, the bread and butter of evolution by natural selection, are concealed. However, over the last 15 years or so, there has been renewed interest in the role of phenotypic plasticity in promoting evolutionary diversification (Nijhout, 1999, 2003b; Pfennig *et al.*, 2010; Espinosa-Soto *et al.*, 2011; Fitzpatrick, 2012).

Waddington (1942) was among the first researchers to suggest that phenotypic plasticity can facilitate evolution through the selection of novel phenotypes. He demonstrated that an environmental stimulus (heat shock) applied to developing *Drosophila* larvae would result in some offspring expressing a particular phenotype (cross-veinless). If these individuals were selected and their offspring (larvae) subject to heat shock, then the frequency of cross-veinless individuals increased. In effect, Waddington (1942) selected for that form of phenotypic plasticity to become more prevalent in the population. However, after relatively few generations of such selection, the environmental stimulus was no longer required to produce the cross-veinless phenotype. Such observations are known as genetic assimilation; the evolutionary reduction in the degree of plasticity such that a character state that was once conditionally expressed depending on the environment becomes

expressed unconditionally regardless of the environment (Fitzpatrick, 2012). This apparent ‘inheritance of an acquired character’ can be explained in terms of natural selection: environmental stress exposes previously hidden genetic variation in developmental pathways that result in the expression of a novel phenotype. This phenotype becomes the subject of selection, which in effect is selection for the novel developmental pathway. Under continual selection, this pathway becomes fixed (genetically assimilated) in the new environment (Pfennig *et al.*, 2010). A similar observation was made by Partridge *et al.* (1994). Rearing *Drosophila* for over 5 years at different temperatures resulted in heritable phenotypic plasticity for body size. Flies that were maintained at low temperature evolved to produce bigger bodies and wings in comparison to flies that were reared at warmer temperatures (Partridge *et al.*, 1994). Of interest, this mirrors the reaction norm; flies reared for one generation at low temperature (16.5°C) were larger than flies reared for one generation at 25°C. Moreover, both the evolved response and the phenotypically plastic response resulted from a change in cell size rather than cell number (Partridge *et al.*, 1994).

It has been suggested that phenotypic plasticity uncovers new genotypic networks that faciliate the stabilization of novel phenotypic expression (Price *et al.*, 2003; Miner *et al.*, 2005; Espinosa-Soto *et al.*, 2011). Moderate levels of phenotypic plasticity are necessary for the survivial of a population under novel environmental conditions in which populations are set to experience peak performance by divergence from the ancestor, as seen in some island colonizers (Price *et al.*, 2003). For example, variation in thermal environments will favour the evolution of generalists capable of maintaining

peak performance across environments, whilst environmental specialists are likely to have a lower peak performance in the face of thermal heterogeneity (Levins, 1968).

Phenotypic plasticity promotes the accumulation of cryptic genetic variation because the phenotypic effects of novel genetic variants are subject to relaxed selection because of their plastic (normal) response in the non-inducing environment (i.e. their effects are usually hidden from selection) (Ancel, 1999, 2000; Sultan & Spencer, 2002; Price *et al.*, 2003; West-Eberhard, 2005b). Phenotypic plasticity promotes the release of this cryptic genetic variation when organisms are exposed to novel environmental conditions. Thus, when exposed to a novel envionment, plasticity produces novel phenotypic variants via the induction of the genetic variants that underlie the genetic pathways. In this way, plasticity can rapidly produce new phenotypic variants in response to environmental change, increasing the likelihood of at least some individuals surviving. Thus, plasticity has been suggested to promote the successful colonization of novel environments (Lefebvre *et al.*, 2001; Mettke-Hofmann *et al.*, 2002).

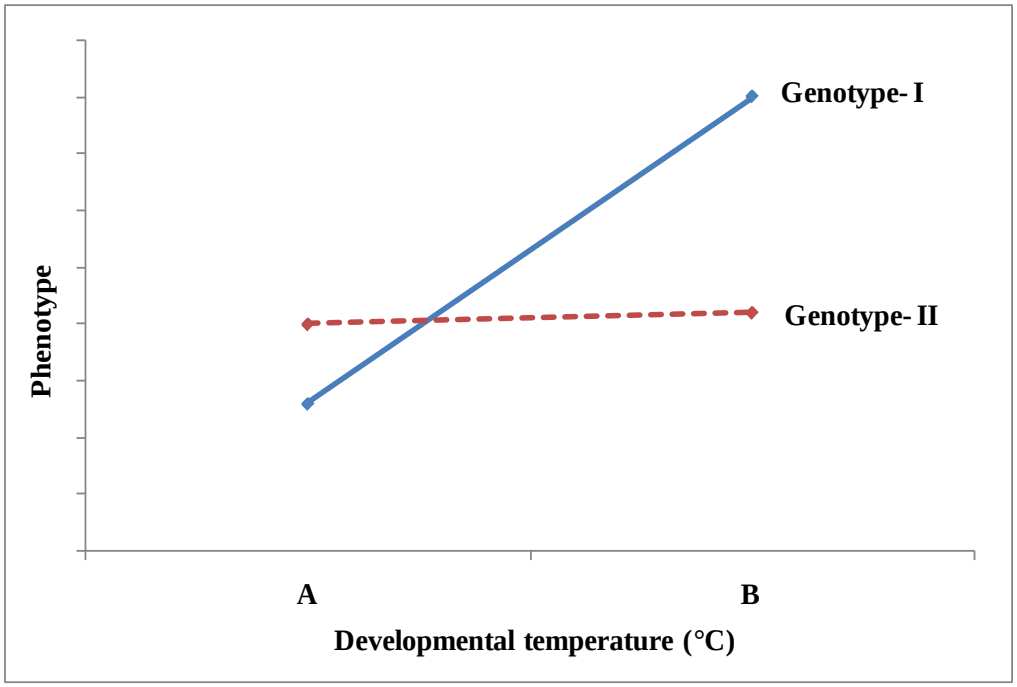


Fig. 2 Production of different reaction norms by two genotypes (I and II) under a variable environment.

Variation in thermal reaction norms can be produced via different classes of trade-off. Thus two genotypes may produce separate reaction norms (Fig. 2). These differences could be caused by different allocation trade-offs such that genotype-I converts more of its finite resources to growth and less to reproduction at higher temperatures than genotype-II. A similar pattern can be achieved via an acquisition trade-off. Thus genotype-I could grow faster at higher temperatures because it acquires resources at a greater rate than genotype-II. Although under this scenario genotype-I avoids an allocation trade-off, it is likely to experience an acquisition trade-off, in which an increase in foraging intensity is likely to result in an increase in the probability of being predated or parasitized. Finally, the same pattern may arise through growth specialization at low (or high) temperatures, such that genotype-II is specialized to grow at low temperatures and genotype-I at high temperatures, but face a specialist-generalist trade-off (Angilletta *et al.*, 2003). This latter

trade-off appears to arise through the structure and function of enzymes. An enzyme with greater conformational stability functions best at high temperatures whilst an enzyme with less conformational stability functions better at lower temperatures (Angilletta *et al.*, 2003). In the common killifish (*Fundulus heteroclitus*), allozymes of lactate dehydrogenase ($LDHB^a$ & $LDHB^b$) enable thermal specialization of swimming endurance. $LDHB^b$ works better than $LDHB^a$ at low temperatures and vice-versa. Fish that are homozygous for $LDHB^b$ sustained greater swimming speeds at 10°C than those homozygous for $LDHB^a$ (Powers & Schulte, 1998).

Polyphenisms are alternative seasonal morphs (distinct phenotypes that arise from a single genotype) such as the changes in pigmentation seen in some insects (Nijhout, 2003a; Suzuki & Nijhout, 2006). Polyphenisms are considered a form of adaptive, discontinuous phenotypic plasticity. For example, in the butterfly (*Araschnia levana*), different generations produce seasonally different forms due to development taking place under different environmental conditions (Nijhout, 2003a). Polyphenisms take place in nature due to discrete developmental switches but most polyphenisms derive from discontinuous environmental effects on the regulatory hormonal mechanisms that underlie the reaction norm and produce the phenotype (Nijhout, 2003a). It has been suggested that polyphenisms could allow individuals to occupy new niches and possibly become successful colonizers (Whitman & Agrawal, 2009).

Adaptive plasticity is ecologically important because it enables individual genotypes to thrive and successfully reproduce under different environmental conditions, playing a major role in the ecological distribution of

organisms (Sultan, 1995, 2000, 2003; Relyea, 2004). In this context, phenotypically plastic responses present a greater potential for evolutionary change than 'classic' genetic mutations, because 'environmentally induced change' in phenotypic expression can induce heritable changes within a population, across numerous individuals, in a short time span: in other words phenotypic change via plasticity can lead to genetic diversification in a very short period of time (West-Eberhard, 2005a). By comparison, a single mutation initiates itself in just one organism and takes several generations to spread within the population before phenotypic expression can result in genetic diversification and eventually phenotypic diversification (West-Eberhard, 2005a). Variable expression of phenotypes by changing the environment during development could provide the basic raw material on which sexual selection could act. For example, in *D. melanogaster*, variation in larval density induced size differences in body size and affected the expression of a range of reproductive traits; copula duration, male share of paternity in a competitive environment, fecundity and latency to mate (Edward & Chapman, 2012).

1.6 Phenotypic plasticity in primary reproductive traits

Phenotypic plasticity in primary reproductive traits has been reported in a few cases. Relative to body size, barnacles (*Balanus glandula*), have the longest penis size of any animal, approximately eight times their body length (Neufeld & Palmer, 2008). On wave-exposed shores, barnacles have stouter, shorter penises in comparison to those individuals found in protected bays. This is a phenotypically plastic response to maintain control over the intromittent organ

during turbulent tidal flows that are associated with exposed rocky shores (Neufeld & Palmer, 2008). Copulation in this sessile species is a trade-off between reaching out to potential mates and controlling the longer-penis. In *Drosophila mediopunctata*, flies reared at 16.5°C had bigger absolute aedeagi than those reared at 20°C (Andrade *et al.*, 2005). Similarly, larval rearing temperature has been shown to affect the structure of the male genitalia of *Anopheles albimanus* (Wied), with the male aedeagus being longer when larvae were reared at 22°C in comparison to those reared at 30°C (Hribar, 1996). In the chironomid midge (*Procladius choreus*), seasonal variation in temperature induced variation to the size of male genitalia (Kobayashi, 1998), whilst in Calopterygid damselflies, males emerging later in the season tend to have smaller genitalia (Roff, 1980, 1986; Córdoba-Aguilar, 2009). Males with wider aedeagi are more effective at displacing sperm from the female spermatheca (Córdoba-Aguilar, 2009). In the yellow dung fly (*S. stercoraria*), flies that emerge later in the season eclose as adults with smaller testes and as a consequence, produce fewer sperm thereby affecting success at sperm competition (Ward & Simmons, 1991). Zera *et al.* (1998), studied the role of juvenile hormone (JH) on ovarian investment and the inducibility of flight morphs in adult *Gryllus assimilis.* They demonstrated that following the administration of a dose of JH, adult *G. assimilis* exhibited reduced flight muscles and an increase in the size of the ovaries compared to control crickets. The trade-off between larger ovaries and shorter flight muscles is thought to be due to an increased allocation of nutrients to reproduction.

Within several taxa, egg size increases at cooler environments. Female *D. melanogaster*, produced larger eggs when reared at 16.5°C in comparison

to those reared at 25°C (Azevedo *et al*., 1996) and in the yellow dung fly, *S. stercoraria*, females laid larger eggs at cooler temperature both under field and laboratory conditions (Blanckenhorn, 2000). Similar results have been reported in the fruit feeding butterfly (*Bicyclus anynana*) (Fischer *et al*., 2003a).

In reptiles, developmental temperature can affect the outcome of sex determination. This is the case in some lizards, all crocodiles and most turtles as they lack genetic sex determination (Gilbert, 2000; Valenzuela, 2001; Warner & Shine, 2008). During embryonic development, the temperature that the embryos experience determines the sex of the individual by influencing gonadal differentiation, i.e. formation of testes/ovaries (Pieau, 1996). In numerous oviparous reptilian species this happens when temperature disrupts the expression of key hormones (e.g. oestrogen) that are responsible for the developmental trajectory the embryo takes. In some turtles, the biosynthesis of oestrogen under variable thermal regimes has been shown to be associated with the gene expression of aromatase inhibitors that affect the sex of the offspring (Pieau, 1996). Developmental modifications to the reptilian embryo (i.e. Environmental sex determination) is thought to be adaptive in nature as it relates to the relative fitness of male and female offspring developing at particular temperatures (Charnov & Bull, 1977; Crews *et al.*, 1994; Pieau, 1996; Valenzuela, 2001).

The semelparous Indian meal moth (*Plodia interpunctella*), exhibits phenotypic plasticity in testes size and sperm number as a result of sensitivity to population density during larval development (Gage, 1995). Males reared at high density took longer to develop, had larger relative testes size and

produced more sperm in comparison to those that experienced little larval competition. This was considered adaptive because male larvae reared at lower densities faced a lower risk of sperm competition upon eclosion and hence allocated more resources to migratory and mate-searching mechanisms, whereas the high density males were anticipated to experience a greater risk of sperm competition (Gage, 1995). Of interest, population density also affected female mating frequency; at high densities females mated more frequently thereby increasing the levels of sperm competition (Gage, 1995). Martel *et al.* (2011) showed that nutritional constraints during larval development (host quality) of the egg parasitoid (*Trichogramma euproctidis*), affected adult size which was positively related to reproductive traits such as sperm size and sperm number. Larger males produced more and bigger sperm than smaller males. Male sperm vesicle size and female spermatheca size were positively correlated with adult size.

Blanckenhorn & Hellriegel (2002) report a phenotypically plastic response of sperm size to variation in developmental temperature in the yellow dung fly *S. stercoraria*. In their first experimental replicate, they demonstrated an increase in sperm length with an increase in developmental temperature from 12°C to 18°C and then a decrease in sperm length when larvae were reared at 24°C. In their second experimental replicate in which larvae were reared at only two developmental temperatures, 15°C and 23°C, they found sperm size to increase with increasing developmental temperatures. However, there was a considerable effect of replicate on sperm size: flies grown at 23°C in replicate 2 produced smaller sperm than flies reared at 12°C in replicate 1. Thus, the effect of larval rearing temperature on sperm length appears to be

inconsistent in their study. Minoretti *et al.* (2013) showed that in the land snail, *A. arbustorum,* sperm length was affected by larval rearing temperature with high developmental temperatures (20°C) resulting in reduced sperm length in comparison to those kept at 11°C & 15°C. Similarly, in guppies (*P. reticulata*) higher developmental rearing temperature (30°C) resulted in males with shorter sperm in comparison to males kept at 23°C, 25°C or 28°C (Breckels & Neff, 2013)

Given that studies into the role of sexual selection in the evolution of primary reproductive traits have produced inconsistent results (Barker & Herman, 1976; Giga & Smith, 1987; Fox, 1994; Blanckenhorn, 2000; Hellriegel & Blanckenhorn, 2002; Fischer *et al.*, 2003a, b; Stillwell & Fox, 2005; Bounds *et al.*, 2010; Eberhard, 2010), it is surprising that few studies have examined the effect of developmental temperature on sperm size (Blanckenhorn & Hellriegel, 2002; Minoretti *et al.*, 2013; Breckels & Neff, 2013). Previous studies into the role of developmental environment on sperm size have focused on nutrients or larval density, both of which appear to have either no or only a small effect on sperm size (Gage & Cook, 1994; Hellriegel & Blanckenhorn, 2002; Amitin & Pitnick, 2007; Gay *et al.*, 2009; but see Martel *et al.*, 2011). In contrast, temperature appears to affect egg size (Azevedo *et al.*, 1996; Fischer *et al.*, 2003a) and sperm size (Blanckenhorn & Hellriegel, 2002; Breckels & Neff, 2013 Minoretti *et al.*, 2013). Therefore, the aim of this study is to investigate the effects of the thermal environment on the phenotypic expression of sperm size and sperm function in *C. maculatus*.

This beetle is an important economic pest species of grains and legumes, infesting crops both in storage and in the field (Beck & Blumer

2011). After successfully mating, females glue their fertilized eggs to the surface of the bean, and larvae burrow directly into the seed where they feed on the embryo and endosperm of the bean. The developing larvae go through four larval moults prior to pupation (Devereau *et al.*, 2003; Beck & Blumer, 2011). Post emergence, the adults readily mate and the life-cycle is repeated. Adults do not require food or water and live for approximately two weeks. At ~ 30°C the life cycle takes ~ 3 weeks to complete (Beck & Blumer, 2011).

1.7 Thesis aims & objectives

Here, I aim to investigate the effects of variation in the thermal environment on sperm structure and function in *Callosobruchus maculatus* (F). Specifically to:

- Determine the effect of larval rearing temperature on larval growth and development.
- Investigate the effect of larval rearing temperature on the expression of sperm length.
- Determine when, during larval development, temperature affects the expression of sperm length.
- Investigate the effect of larval rearing temperature on reproductive behaviour (copula duration).
- Determine the effect of larval rearing temperature on male reproductive performance during sperm competition.
- Discuss the mechanisms and consequences phenotypic plasticity in primary reproductive traits and recommendations for future studies.

2. The effect of temperature on larval growth and development

2.1 Introduction

The growth and development of ectothermic species is known to be affected by temperature (Stearns, 1992; Davidowitz & Nijhout, 2004; Atkinson *et al.*, 2006; Angilletta, 2009). Among the most important and widespread effects of environmental temperature is its influence on the plastic expression of phenotypes (Stearns & Koella, 1986; Angilleta, 2009). Phenotypic plasticity is the property of an organism to react to environmental heterogeneity in space and time, exhibiting distinct phenotypes for a given genotype - i.e. a reaction norm (Pigliucci 2001; Price *et al.*, 2003; Angilletta, 2009; Muschick *et al.*, 2011).

A widespread occurrence, termed the 'temperature-size rule', is that low temperatures experienced during development generate a reaction norm wherein organisms exhibit larger adult body size as a result of delayed maturity (Angilletta & Dunham, 2003; Davidowitz & Nijhout, 2004; Atkinson *et al.*, 2006; Angilletta, 2009; Nijhout, 2010). Under natural conditions many endothermic organisms mature at larger body sizes at higher latitudes or altitudes; a phenomenon known as Bergmann's rule or Bergmann's clines (Bergmann, 1847; Angilletta & Dunham, 2003; Angilletta *et al.*, 2004a). Although the rule was originally conceptualized to apply to endothermic species, Bergmann's clines have since been reported in several ectotherms (Lindsey, 1966; Pincheira-Donoso *et al.*, 2008). Both the temperature-size rule and Bergmann's rule give rise to a negative relationship between the temperature experienced during development and body size (Meiri *et al.*,

2007). Bergmann's rule is usually interpreted as an adaptive response wherein larger animals that experience cold temperatures lose less heat due to a reduced surface area to volume ratio (Mayr, 1956). However, this cannot be the case for ectotherms and some authors have postulated that larger body size correlates with food availability, primary productivity and/or geographical factors other than temperature (Rosenzweig, 1968; Blanckenhorn, 1999; see Meiri *et al.,* 2007). A number of empirical studies have supported the temperature size rule, with seasonality of temperature being one of the most common aspects studied in relation to body size plasticity in endotherms (Graves, 1991; Freckleton *et al.*, 2003; Meiri & Dayan, 2003). However, Bergmann's rule, i.e. maintenance of larger body sizes at low temperature, does not universally apply to all ectotherms (Meiri & Dayan, 2003; Adams & Church, 2007; Pincheira-Donoso *et al.*, 2007, 2008). The temperature size rule raises two questions: i) What mechanisms bring about the relationship between temperature and size and ii) is this relationship adaptive?

To attain a larger body size the organism needs to continue to grow beyond the normal period of development. Should temperature affect growth and development equally, an organism would simply reach the same size but take longer or shorter at cool and warm temperatures respectively (Nijhout, 2003b). Thus, growth and development need to de-coupled in order to produce temperature dependent phenotypic plasticity (Forster & Hirst, 2012). In holometabolous insects, growth is terminated at the onset of pupation or metamorphosis, which is controlled by an underlying endocrine cascade (Chapman, 1998; Davidowitz *et al.*, 2004). Development time and ultimately body size is controlled by, i) growth rate, ii) the timing of cessation of juvenile

hormone secretion and iii) the timing of ecdysetroid secretion necessary for pupation. Thus, stage-specific hormonal synthesis is responsible for the termination of larval growth and the onset of metamorphosis (Davidowitz & Nijhout, 2004; also see Parker & Johnston, 2006).

Differences in body size can come about via differences in cell size, cell number or a combination of the two. In *D. melanogaster*, body size (or organ size) is the product of both cell size and cell number (Alpatov, 1930; Robertson, 1955, 1959; Partridge *et al.*, 1994; Partridge & French, 1996; De Moed *et al.*, 1997; Azevedo *et al.*, 2002). For example, strain differences in the size of *D. melanogaster* were attributed to differences in cell number, indicating cell number to be influenced by an underlying genetic factor (Robertson, 1955, 1959). By contrast, inbred lines reared at high temperatures produced smaller adults due to a reduction in cell size but not cell number (Robertson, 1955, 1959; Partridge & French, 1996; De Moed *et al.*, 1997). Therefore, cell size and cell number appear to be controlled by different regulatory mechanisms that respond independently to genetic and environmental variation. However, body size, just like any other tissue or organ, is difficult to explain in terms of cell size and cell number and body size is simply the sum total of all the regulatory mechanisms associated with size of other internal appendages (Nijhout, 2003b).

Growing to a smaller size at higher temperatures has been termed a ‘life-history puzzle’ (Sevenster, 1995) because one might expect organisms to exploit the higher growth rates and grow to a larger size, so as to gain the benefits of increased fecundity, longevity and mating success that come with larger body size (Kingsolver & Huey, 2008). There is good evidence that

temperature reaction norms are underpinned by genetic variation (Kingsolver & Huey, 2008) and thus the target of natural selection. Therefore, the question may be rephrased to: Why has natural selection not resulted in the evolution of flat reaction norms? One possibility is that under high temperatures the cost of growing to a large body size outweighs the benefits. A key mechanistic model proposed to account for the TSR (temperature-size rule) is that development rate increases at an increasing rate with temperature, whilst growth rate increases proportionately. In effect growth and development are de-coupled (Forster & Hirst, 2012) and the synchronisation of these rates, across a range of temperatures, may be too costly. Another adaptive explanation is that those individuals that reach sexual maturity faster, gain from 'compound interest'. Effectively, by maturing and reproducing early, individuals produce more descendents than via the alternative strategy of delaying maturity and reaching greater body size and thus greater fecundity. Empirical tests of the adaptive nature of TSR are quite limited (see discussion of Stillwell *et al.*, 2008). Stillwell *et al.* (2008) used artificial selection to create large and small lines of the seed beetle (*Stator limbatus*). They then raised these beetles under cool and hot conditions to the test the hypothesis that the large beetles would be relatively more successful than small beetles under cold conditions. They actually found no difference in the relative fitness of large beetles when reared under a low temperature and thus concluded that increased body size was not an adaptation to cold conditions.

Females of many species exhibit higher rates of fecundity with increasing body size (Roff, 2002) but flies from colder environments may not necessarily experience higher fecundity due to slower growth rates than flies

form warmer environments. Therefore given thermal constraints on life-history traits, adaptive explanations are centered on a cost and benefit analysis (trade-offs) (Atkinson, 1994; also see Atkinson & Silby, 1997). In this regard, the thermal environment acts as an agent of natural selection by influencing the developmental mechanisms proximate to growth rate and final adult size (Noach *et al.*, 1997; Zwaan *et al.*, 2000; Kindleman *et al.*, 2001; Angilletta *et al.*, 2002; Angilletta *et al.*, 2004a; Grant & Grant, 2007; Angilletta, 2009).

For many life-history traits, differences in the function of structural enzymes are brought about by variation in developmental conditions (e.g. temperature) that determine the fate of the thermal reaction norms (Somero, 1995; Angilletta *et al.*, 2003). For example in *D. melanogaster*, greater conformational stability of enzymes resulted in enhanced performance at higher temperatures whilst reduced conformational stability resulted in better performance at lower temperatures (Edgar, 2006; Espinosa-Soto *et al.*, 2011). Thus, plasticity in the expression of enzymes via novel gene regulatory processes under novel environmental conditions can potentially lead to the evolution of new gene regulatory processes involved in enzyme biosynthesis, i.e. the alteration of genetic expression in the process of enzyme biosynthesis (Espinosa-Soto *et al.*, 2011). When phenotypic plasticity is non-adaptive (i.e. pathological), phenotypic change is a direct consequence of thermal constraints on biochemical processes that negatively impact on development (Nylin & Gotthard, 1998; Angilletta, 2009).

Here, I examine the effect of temperature on growth and development in *Callosobruchus maculatus* (Coleoptera: Bruchidae). The main thrust of this thesis is an examination of the effect of developmental temperature on the size

of sperm (see chapter 3 and the references therein; Blanckenhorn & Hellriegel, 2002). Thus, in order to more fully understand the mechanistic basis of plasticity in sperm size, it is necessary to have a better understanding of how variation in developmental temperature affects larval growth and development. It is predicted that decreasing temperature during larval development will increase development time and consequently increase offspring size, in line with a number of insect studies (Chandrakantha & Mathavan, 1986; Stillwell & Fox, 2005, 2007; Stillwell *et al.*, 2007; Stillwell *et al.*, 2008).

2.2 Materials and Methods

2.2.1 Model system and experimental design

The *Callosobruchus maculatus* beetles used in this study came from a large, outbred population (~ 5,000 adults) cultured for 24 generations on moth beans (*Vigna aconitifoli*) in an insectary maintained at 27°C, 32% relative humidity (RH) and a 16L:8D (Light:Dark; hours) photoperiod. The parental stock population originated from Niamey, Niger, and had been kept on black-eyed beans (*Vigna ungliculata*) for more than 100 generations at the University of Lincoln. Moth beans were used instead of larger black-eyed beans because only one adult emerges from each seed, enabling easy control over larval density.

Approximately 1,000 adults (figure estimated by mass) were housed on 200g of moth beans (~ 7,500 beans) for 4h in a 150x15mm Petri dish. The egg-laden beans were separated into five triple vent 110 mm Petri dishes, with approximately 1,200 beans per dish. The eggs were incubated in one of the

five different incubators (Lucky Reptile Herp Nursery II, The reptile shop, UK) maintained at 17°C, 20°C, 25°C, 27°C and 33°C. Development time was measured by taking the mean (± standard error) time taken in days from egg-to-adult eclosion at the different larval rearing temperatures (N= 40 per treatment). To determine changes in larval size, a sample of egg-laden beans were cracked open at intervals (depending on respective temperature) between 8 – 81 days for larval length and 5 – 50 days for head capsule width (head capsule width was not measured for those reared at 20°C). Larval length (mm) and head capsule width (mm) of approximately 10 larvae per sampling point per treatment were measured under an Olympus stereoscopic dissecting microscope [SZH10] linked to an Image analysis workstation (Moticam 2000). In total over 100 larvae were measured. Head capsule width was measured to identify larval instars (Devereau *et al.*, 2003). The measurements were made using ImageJ software using the un-segmented tool. Some (N= 40 per treatment) larvae were allowed to complete full development. In these cases the elytra length of the resulting adults were measured as a proxy of body size (Wilson & Hill, 1989). Mass of individual adult beetle was measured using an electronic balance (Ohaus Analytical plus balance, model no. AP110S) and adults were euthanized 24 – 48h old post eclosion. Prior to the measurement of mass, the beetles were stored in a 0.5ml eppendorf tube in a freezer maintained at -5°C; approximately twenty individual adults were measured per thermal environment.

The parametric data presented here was analyzed for both deviations from normal distributions and for heterogeneous variances between the various treatment groups. To investigate differences in larval traits as a result of

different developing conditions, one-way ANOVA was employed using SPSS version 20 (IBM) and *Post hoc* Tukey test revealed statistically significant ($P<0.05$) differences between groups.

2.3 Results

Development from egg-to-adult was longest at 17°C (87.30 ± 0.1 days), being approximately four times longer than development at 33°C (22.29 ± 0.1 days) (ANOVA: $F_{4,100} = 35698.1$, $P < 0.0001$, Fig. 3). The time taken to reach pupation was longest at 17°C taking on average 56 days in comparison to those beetles reared at 33°C which occurred around day 13 or 14 days (Fig. 4). Similarly, pupation to adult eclosion was approximately 8 times longer at 17°C in comparison to those larvae reared at 33°C. Larval head capsule width (mm) demonstrates 4 peaks (also see Devereau *et al.*, 2003) in size at approximately 0.12 – 0.18 mm, 0.22 – 0.28 mm, 0.4 – 0.46 mm and 0.52 – 0.60 mm respectively, representing the first, second, third and fourth larval instars (Fig. 5). If these are plotted against proportion of time in the larval stage (i.e. from oviposition to pupation) (Fig. 6), larval instar I is present up to ~ 40% of larval development, instar II is between 40% – 55%, instar III is between 55% – 75% and instar IV after ~ 75% of larval development (Fig. 7). A two- way ANOVA comparing adult elytra length in relation to treatment and sex revealed larval rearing temperature to have a significant effect on elytra length ($F_{3,160} = 24.4$, $P < 0.0001$) with those larvae reared at the coolest temperature developing into the largest adults. There was no effect of sex ($F_{1,160} = 2.7$, $P = 0.099$) nor the interaction between larval rearing temperature and sex (ANOVA: $F_{3,160} = 0.8$, $P < 0.484$, Fig. 8) on adult elytra length. In

terms of weight, a Two-way ANOVA revealed a significant effect of developmental temperature ($F_{3,152}$ = 42.0, P < 0.0001), sex ($F_{1,152}$ = 52.0, P < 0.0001) and the interaction between developmental temperature and sex ($F_{3,152}$ = 2.9, P < 0.036, Fig. 9) on adult mass. Thus, females were heavier than males and adults were larger at lower developmental temperatures (in accordance with the temperature-size rule) and the extent of size dimorphism tended to be greatest at intermediate temperatures.

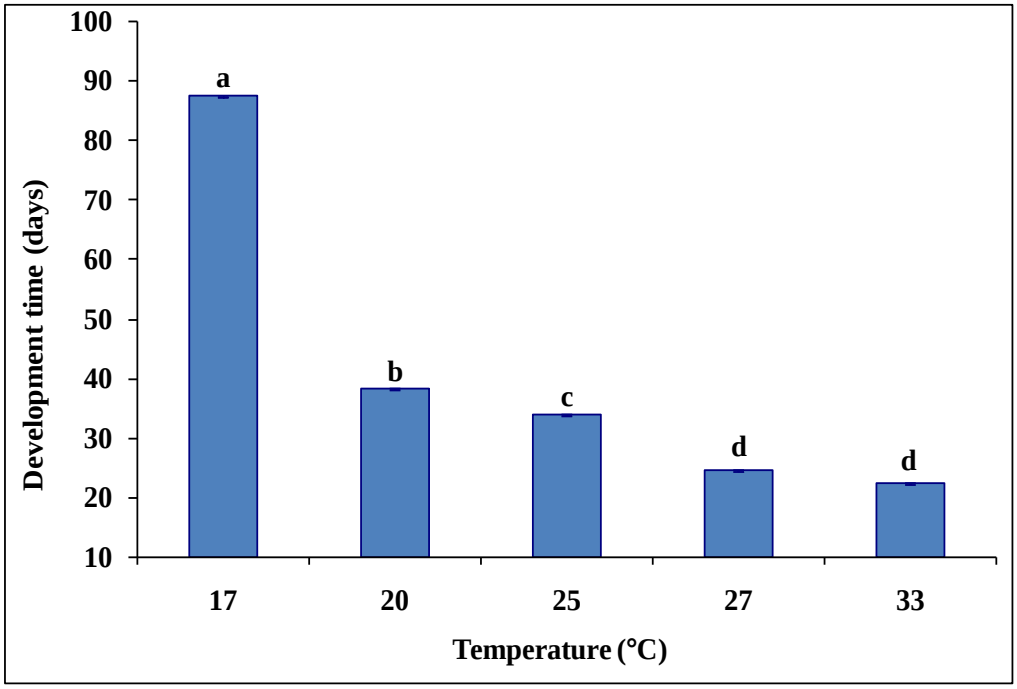


Fig. 3 Mean ± developmental time (days) from egg-to-adult at the different rearing temperatures. Different letters above bars indicate significant differences (P<0.05) following *Post-hoc* Tukey test.

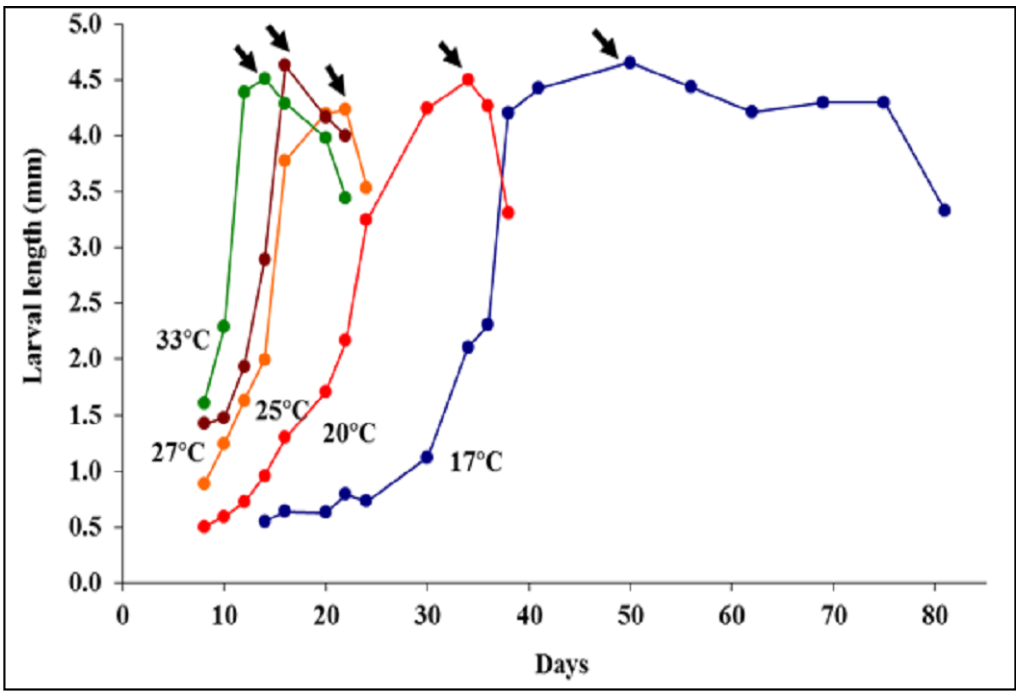


Fig. 4 Larval length in relation to time since oviposition (days) at the 5 different rearing temperatures. Arrows indicate pupation. Each closed circle represents a mean value (N=10).

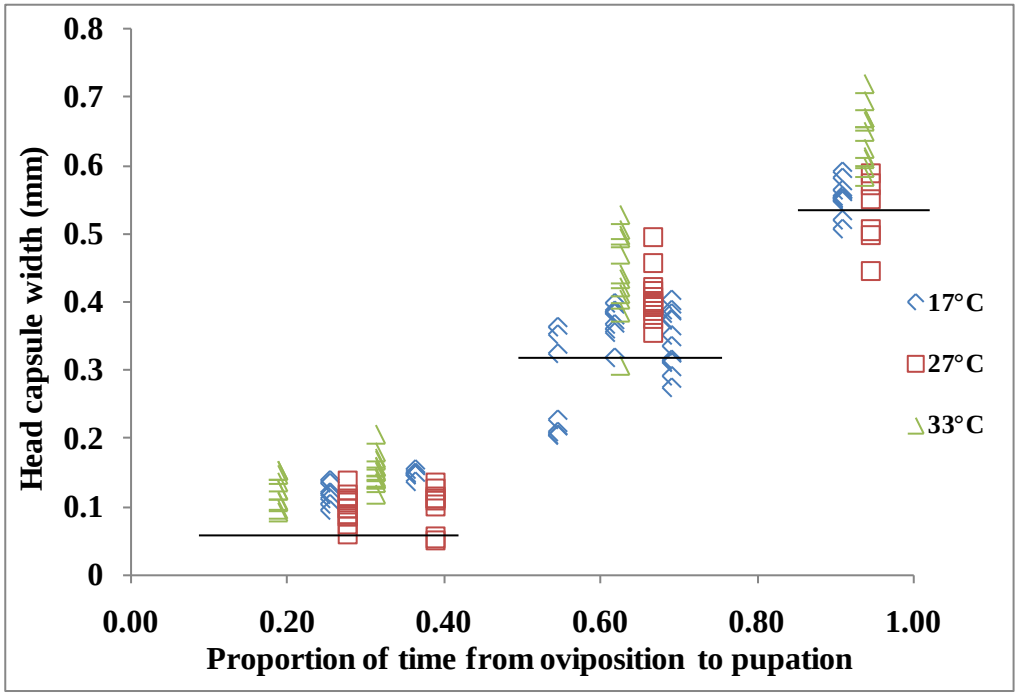


Fig. 5 Larval head capsule width (mm) in relation to proportion of time from oviposition to pupation at the different larval rearing temperatures.

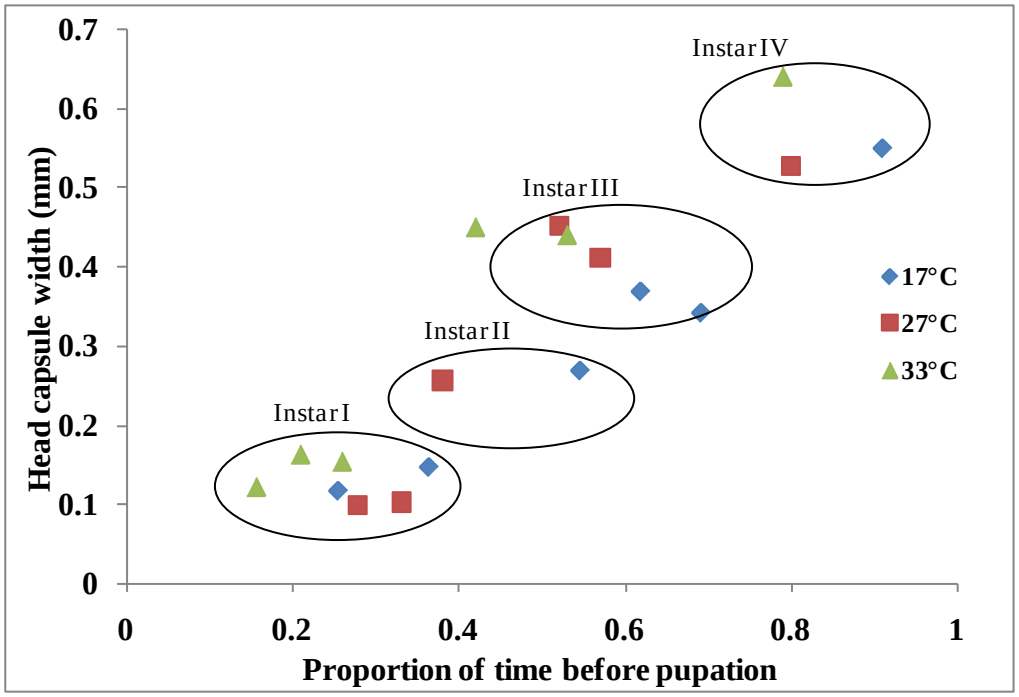


Fig. 6 Scatter plot of head capsule width (mm) in relation to proportion of time before pupation at the different developmental temperatures, 17°C (diamonds), 27°C (squares) and 33°C (triangles).[1]

[1] Each shape within a cluster (Instar, I, II, III and IV) are head capsule width values (mean ± SD, mm) measured at different time points (days). For instance, the two red squares within Instar I cluster show head capsule measurements on different days.

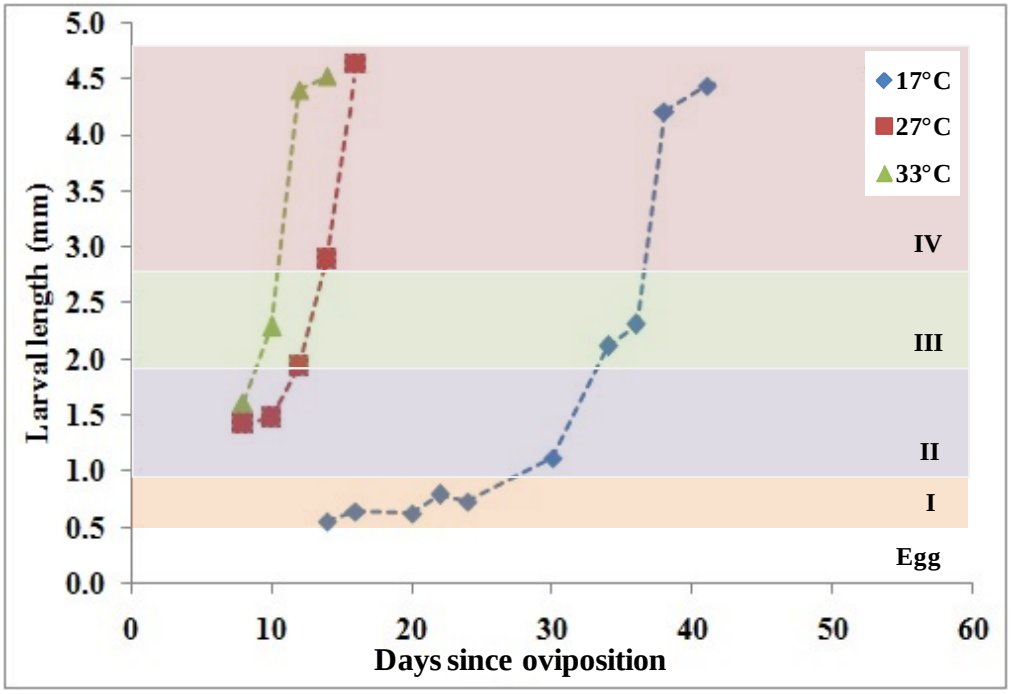


Fig. 7 Larval length (mm) in relation to days since oviposition at the three developmental temperatures which represents the different larval instars taking place; 17°C (diamonds), 27°C (squares) and 33°C (triangles).

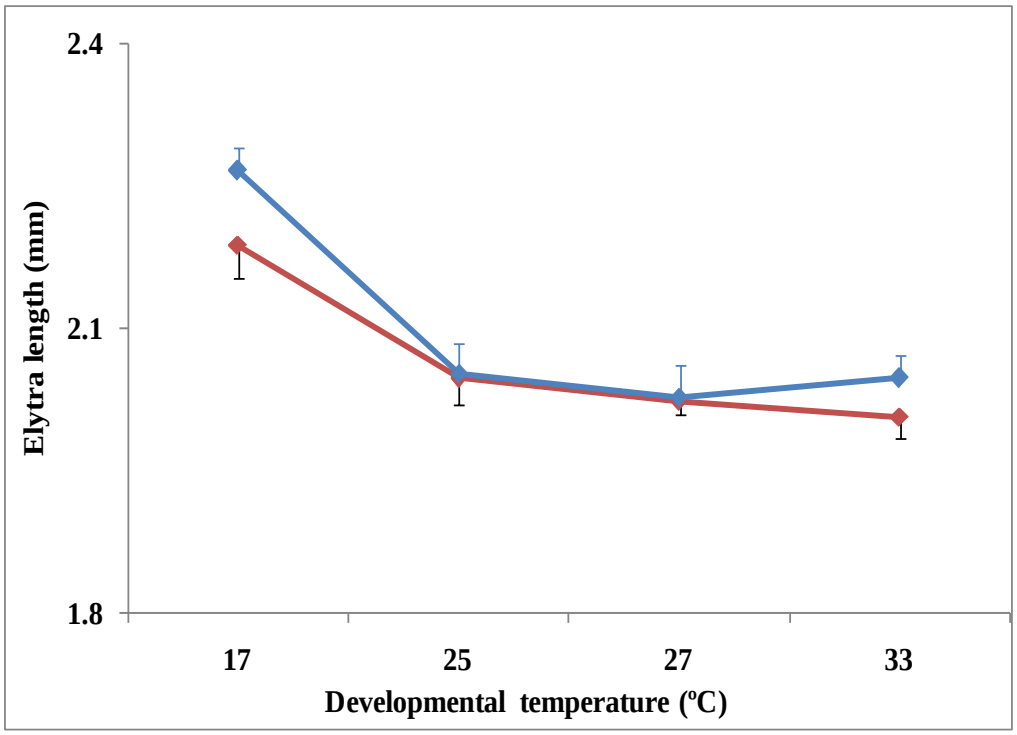


Fig. 8 Mean ± standard error, elytra length of male and female adults when larvae were reared at different temperatures. Male (red line) and female (blue line).

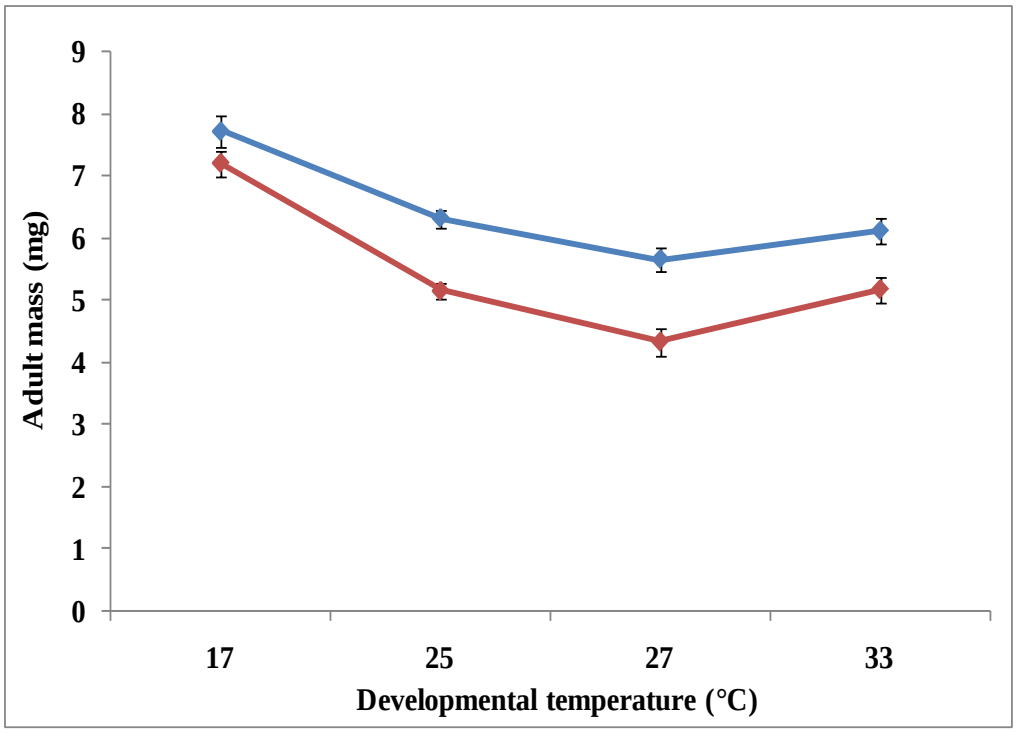


Fig. 9 Mean ± standard error adult mass of males (red line) and females (blue line) when larvae were reared at different temperatures.

2.4 Discussion

Body size in *Callosobruchus maculatus* follows the temperature-size rule, with lower temperatures resulting in longer development times and larger adult offspring. Smaller ectotherms (both vertebrates and invertebrates) consistently conform with the temperature-size rule (see Pincheira-Donoso, 2010), although the rule does not always hold true for larger ectotherms, e.g. some snakes, lizards and turtles (Pincheira-Donoso, 2010) because the inverse heating-rate and body mass relationship make it disadvantageous both physiologically and ecologically to maintain larger body sizes in colder environments (Pincheira-Donoso, 2010). Here beetles reared at 17°C were larger than those reared at higher temperatures. Larger size could provide better heat conservation mechanisms, greater food reserves, resistance to starvation and/or represent trade-offs associated with life-history processes such as duration and timing of hormonal biosynthesis (Atkinson, 1994; Nijhout, 2003b; Davidowitz & Nijhout, 2004; Edgar, 2006; Pincheira-Donoso, 2010).

Larval growth and development and hence body size depend on two factors; food and temperature (see. Blanckenhorn, 1999). In holometabolous insects, growth is observed until metamorphosis and some larval imaginal cells die once metamorphosis (cell differentiation) is attained (French *et al.*, 1998). With regard to nutrition, rearing temperature could affect the amount of food consumed during the larval stages, the efficiency of assimilation, the conversion of food into tissue (body) and the balance between the production of epidermis (which determines the adult size) and internal tissue associated with the adult body (see Chandrakantha & Mathavan, 1986; Stillwell & Fox,

2007; Stillwell *et al.*, 2008). Neat *et al.* (1995) found that *Drosophila* larvae kept individually at 16.5°C with depleted amounts of food emerged as smaller adults (smaller: suggested less food was consumed) than those kept at 25°C with adequate food, suggesting body size to be associated with greater levels of food intake (but see Nijhout, 2003b; Reiskind & Zarrabi, 2012). Neat *et al.* (1995) suggested that temperature affected the modulation of body size via changes in both cell size and cell number. This potentially took place during the latter stages of mitosis whereby the lack of availability of nutrients resulted in the epidermal cells remaining smaller in size and fewer in number (French *et al.*, 1998; Nijhout, 2003b).

The study here shows a relationship between developmental temperature and body size. As in the majority of ectothermic species, *C. maculatus* demonstrate slower growth, and mature at a larger size in colder environments, as a direct influence of the thermal environment on the putative body size reaction norm (Angilletta, *et al.*, 2004b). Phenotypic plasticity could play a major role in the maintenance of variation in body size via natural selection for unique genetic regulatory circuits exposed during environmental stress that could facilitate adaptive evolution (Partridge *et al.*, 1994; also see Espinosa-Soto *et al.*, 2011). Evolved differences in body size in response to the thermal environment appear to coincide with the body size-temperature reaction norm suggesting genetic assimilation (Partridge *et al.*, 1994; also see Espinosa-Soto *et al.*, 2011). There is some evidence to suggest that through prolonging growth and delaying maturity to achieve larger body size in colder environments could result in increased longevity and allow greater investment into bigger but fewer eggs (Via *et al.*, 1995; Heino & Kaitala, 1999; Angilletta

et al., 2004b). However, Stillwell *et al.* (2008) selected for large and small body size in *S. limbatus* and found that larger body size was not adaptive at lower temperatures. In essence, the relative fitness advantage of being large at low temperatures was maintained at higher temperatures, i.e. big is good but not better at low temperatures (also see Arendt & Fairbairn, 2012).

The body size plasticity found in the present study is in contrast with Schutze & Clarke (2008) who report that in the herbivorous Eucalyptus beetle (*Paropsis atomaria* Olivier), adult size decreased at increasing altitudes (lower temperatures), exhibiting a reverse Bergmann cline. They suggested that the pattern could be the result of a developmental adaptation to season length as the species is a largely heat-adapted species in their natural environment where they experienced a lesser degree of exposure to low thermal conditions (lack of micro-climate fluctuations). Thus, by subjecting them to cold conditions they experienced developmental stress whereby they attained smaller sizes in comparison to those reared under normal conditions.

In *Aedes albopictus*, hotter rearing temperatures resulted in smaller wing sizes, a proxy for adult size, but smaller adults were shown to have a greater body mass than individuals reared at cooler temperatures (Reiskind & Zarrabi, 2012). In *A. albopictus*, the reason for smaller adults to be heavier was interpreted to be due to mosquito larvae being able to feed on microbes present in the medium more freely at a higher rate. The higher feeding rate was triggered by a greater rate of microbial multiplication, and thus an abundant supply of food. Higher feeding rate enabled the smaller adults to store more non-structural energy in the form of glycogen whilst larval development was still under the influence of constraints imposed by thermal

environment on adult body size (Reiskind & Zarrabi, 2012). In my results, I found that females had a greater mass (especially those reared at 25°C to 27°C), but not a greater elytra length than males. Chandrakantha *et al.* (1987) found total food consumption, egg mass and energy content of the egg in *C. maculatus* decreased with increasing temperatures. Larvae reared at 35°C consumed less food, as revealed by measuring the mass of the larval instars and the unfed remains of the seed in comparison to larvae reared at 20°C or 25°C; *C. maculatus* larvae reared on cowpeas *(Vigna ungiculata)*, consumed 444 Joules/larvae at 20°C in comparison with 367 Joules/larvae at 35°C (Chandrakantha *et al.*, 1987). This indicates food consumption was directly affected by larval rearing temperature which in turn affected final adult size (Chandrakantha *et al.*, 1987; see Stillwell & Fox, 2007).

The sexual size dimorphism (SSD) of males and females observed in this study increased at the temperature extremes. Both elytra length, a surrogate of adult body size (mm) and adult mass (mg) showed varied response to larval rearing temperatures (Figs. 8 & 9). SSD for elytra length was seen to be greatest at the extreme temperatures (17°C and 33°C); however SSD for adult mass was greatest at the intermediate temperatures (25°C and 27°C). Similarly, Stillwell & Fox (2007) showed that rearing temperature affected the gender-induced plasticity in body in response to rearing temperature in the South Indian strain of *C. maculatus*. They suggested that male body size was more sensitive to (rearing) temperature fluctuations than female body size and SSD was greatest at high temperatures 30°C but not at the lower temperature (20°C). This deviation from the results presented in this thesis is open for speculation. One possible explanation suggested by Stillwell

& Fox (2007) was that the differences in attaining fitness optima for any trait in a given environment will differ between populations that are regulated by developmental canalization (Stillwell & Fox, 1997). Furthermore, it has also been suggested that the degree of sexual size dimorphism patterns could also be shaped by the differences in larval mortality in response to rearing temperatures (Blanckenhorn, 1997). Both the degree and direction of SSD in body size of the sexes at higher temperatures were comparable.

In multicellular organisms, the ratio between adult size and progeny size is not constant at different developmental temperatures. In unicellular organisms this ratio is constant as a result of constraints on binary division (Forster *et al.*, 2011). However, in multicellular organisms, growth and development rates differ at different temperatures, (g/D), wherein body size is affected by becoming progressively smaller at elevated temperatures relative to the rate at which growth takes place. Across taxa (e.g. insects, snakes, fish, birds), the observed patterns of body-size variation in relation to temperature is thought to be due to the unequal effects of temperature on growth and development (see Forster *et al.*, 2011a). Therefore, differences in adult size could be due to differences resulting from the divergence of development time and growth rate (e.g. arthropods, see Blanckenhorn *et al.*, 2007).

Underlying physiological mechanisms have been known to affect growth rate and with it the expression of body size (Stillwell *et al.*, 2010). Variations to body size in *C. maculatus* that was observed in this study may be due to changes in hormonal titres that control and regulate body size in response to larval rearing temperature (see Stillwell *et al.*, 2010). The expression of an Insulin-like growth factor (IGF) at different intervals has

been shown to influence cell growth and cell size during larval development in the tobacco hornworm (*Manduca sexta*), whereby the timing of its synthesis is affected by developmental temperature. This subsequently affects the rate of cell growth and final adult size (Edgar, 2006). Thus, because insects do not grow as adults, the final body size is a product of duration of growth phase and the rate at which growth takes place (Edgar, 2006).

The growth curves appear (Fig. 4) quite normal in comparison to other studies (Chandrakantha & Mathavan, 1986; Chandrakantha *et al.*, 1987; for marine copepods see Forster *et al.*, 2011b). The pattern of the growth curves found in the present study demonstrates that at hotter rearing temperatures the accumulation of mass (growth rate) increases. However, growth rate is outpaced by developmental rate i.e. the formation of the different larval stages. Thus, growth rate and development rate remain partially decoupled at the different thermal environments (also see Forster *et al.*, 2011b). Forster *et al*. (2011b) showed that across species of marine copepods both growth rate and developmental rate remain decoupled at different temperatures whereby developmental rate follows a convex curve and growth rate followed either a linear or concave curve to temperature. It was suggested by van der Have & de Jong (1996) that growth and development are associated with different mechanistic processes. Growth rate is determined by the rate at which protein synthesis occurs and DNA replication determined development rate as per their model thus fundamentally differing in the size of the biomolecules involved in these two processes (van der Have & de Jong, 1996). The rate at which protein synthesis occurs is limited by the rate of diffusion of biomolecules within the cell (e.g. ribosomal units) however, DNA replication,

which involves the enzyme DNA polymerases is limited by the time taken for the enzyme to attach to the DNA template. They suggest that, at any given temperature diffusion of biomolecules is not as sensitive as enzymatic processes taking place within the cell (van der Have & de Jong, 1996) as diffusion limits the rate of protein synthesis whereas enzymatic processes limit the rate of DNA replication. In the present study, the rate at which growth takes place is out of sync with the metabolic rate throughout ontogeny similar to those seen in other holometabolous insects (see van der Have & de Jong, 1996). Therefore, adults attain smaller sizes (via faster development) at hotter rearing temperatures when compared to those reared at lower temperatures.

With regards to larval development, the data presented here is similar to those of Devereau *et al.* (2003) who found *C. maculatus* larvae to pass through four larval instars prior to pupation. In their study of a Ghanaian population of a *C. maculatus* reared on cowpeas (*Vigna ungiculata*) at 27°C, instar I was evident between 5 – 10 days post oviposition, instar II ~ 8 – 12 days, instar III ~ 11 – 15 days and instar IV ~ 13 – 20 days post oviposition. Adult emergence was between 24 – 30 post oviposition. Based on these data (and those of Devereau *et al.*, 2003) it appears that larval instar I, II, III and IV occur at approximately 20 – 40%, 40 – 55%, 55 – 75% and 75+ % of development time, irrespective of developmental temperature.

To conclude, the developmental time of *C. maculatus* under different rearing conditions is consistent with the results of Chandrakantha & Mathavan (1986) & Chandrakantha *et al.* (1987). At cooler temperatures development takes longer and adults eclose at a larger size. Although these findings are interesting in their own right, they were not gathered to test the temperature-

size rule. Rather these data are necessary to help understand the stage-specific responses of developing larvae to changes in the thermal environment as presented in chapter 4.

3. The effect of thermal environment on the expression of sperm length

3.1 Introduction

Across species, primary reproductive traits are highly varied (Pitnick *et al.*, 2009). For example, in the porcupine (*Hystrix africaeaustralis*), sperm are just 28µm long whilst in (*Drosophila bifurca*), sperm measure 5.8cm in length, approximately 20 times the size of the fly (Pitnick *et al.*, 1995). Although the underlying selection pressures that drive the evolution of this diversity are poorly understood, a number of studies have found both inter- and intraspecific variation in sperm length to be associated with various ecological factors (Ward & Hauschteck-Jungen 1993; Oppliger *et al.*, 1998, 2003; Morrow & Gage 2000, 2001a).

Interspecific variation in sperm length has been shown to coevolve with the dimensions of the female reproductive tract. For example, a positive relationship between sperm length and the length of the spermatheca has been reported in featherwing beetles (Ptiliidae) (Dybas & Dybas, 1981) and the length of the seminal receptacle in *Drosophila* (Pitnick *et al.*, 1999). In stalk-eyed flies (Diopsidae), dung flies (Scathophagidae), moths (Lepidoptera) and bruchid beetles there is a positive relationship between sperm length and spermathecal duct length (Presgraves *et al.*, 1999; Morrow & Gage 2000; Minder *et al.*, 2005; Rugman-Jones & Eady, 2008). Sperm length and sperm storage tubule length are also positively associated across species of birds (Briskie & Montgomerie, 1992). At an intraspecific level, Amitin & Pitnick (2007) have reported sperm length to be positively associated with seminal receptacle length in *D. melanogaster*. In a similar vein, Miller & Pitnick

(2002) found that increasing the length of the ventral seminal receptacle (the female sperm storage organ) via artificial selection, resulted in a corresponding increase in sperm length. A popular explanation for this male/female coevolution is postcopulatory sexual selection, especially cryptic female choice, such that the female reproductive environment sets the rules by which post-copulatory sexual selection is played out (Eberhard, 1996) and this exerts selection on the primary reproductive traits of males. However, the exact mechanisms by which this happens is still to be explained (Hosken & Stockley, 2004).

Within species variation in sperm length has been shown to be positively related to male body size (a proxy for male condition) in the guppy (*Poecilia reticulata*) (Skinner & Watt, 2007), whilst in the dung beetle (*Onthophagous taurus*) smaller males had longer sperm (Simmons & Kotiaho, 2002), and in *Drosophila bifasciata* there was no relationship between sperm size and male size (Kurokawa *et al.*, 1974). A reduction in food quality or quantity also had no effect on sperm size nor the ratio of apyrene and eupyrene sperm in the Indian meal moth (*Plodia interpunctella*) (Gage & Cook, 1994). Studies into the effects of alternative mating strategies on sperm size have produced inconsistent results (reviewed by Birkhead & Møller, 1998) and experimental evolution studies in *D. melanogaster* (Pitnick *et al.*, 2009) and *C. maculatus* (Gay *et al.*, 2009), where postcopulatory sexual selection was altered by enforcing monogamy, showed no effect on sperm length (Hosken *et al.*, 2001). Thus, within species there are few if any, consistent ecological covariates of sperm length.

Many studies on spermatogenesis in insects have revealed sperm size

to be determined relatively early during development e.g. Mecoptera, Lepidoptera, Isoptera, Diptera (Gassner *et al.*, 1972; Witalis & Godula, 1993; Grandi, 1994; Cepeda-Palacios *et al.*, 2001). However, very little is known about the mechanisms that are responsible for sperm size plasticity or whether the plasticity is adaptive (Morrow *et al.*, 2008). Numerous studies have reported phenotypic plasticity of egg size (Aezevedo *et al.*, 1996; French & Partridge, 1996 Blanckenhorn, 2000; Fischer *et al.*, 2003a,b; Liefting *et al.*, 2010). Fischer *et al.* (2003a) showed that female *Bicyclus anynana* butterflies exposed to cold temperatures during oviposition (20°C), laid bigger but fewer eggs than females reared under hot conditions (27°C). This temperature mediated plasticity in egg number/size, resulted in i) enhanced offspring survival when individuals were switched from cool to hot environments in comparison to those individuals switched from hot to cool environments and, ii) a higher rate of hatching of the bigger eggs from the cold environment when eggs were transferred to hotter environments, suggesting bigger eggs could be adaptive at low temperatures (Fischer *et al.*, 2003b; see Fox, 1994).

In the bivoltine scorpion fly (*Panorpa vulgaris*), sperm size was related to season (Vermeulen *et al.*, 2009). First annual generation males were larger and produced larger sperm than males derived from later generations. However, in this species sperm length is not a simple function of body size because second generation males kept in groups but fed *ad libitum* were larger but had smaller sperm than second generation males kept singly but food deprived (Vermeulen *et al.*, 2009). Larval density had no effect on sperm size in the Indian meal moth (*P. interpunctella*), male larvae reared at high densities eclosed into adults with relatively larger testes and could produce

greater number of sperm, but sperm size was unaffected (Gage, 1995). A similar pattern has been reported in the house mouse (*Mus musculus domesticus*); males demonstrated phenotypic plasticity in daily sperm production and the total number of sperm produced in response to social environments (Ramm & Stockley, 2009). Sperm production in this species was dependent on the number of rivals present and the perceived risk of sperm competition.

To date, only three studies have reported an effect of larval rearing temperature on sperm size: the study by Blanckenhorn & Hellriegel (2002) on the yellow dung fly (*S. stercoraria*); the study by Minoretti *et al.* (2013) on the land snail (*A. arbustorum*); and the study by Breckels & Neff (2013) on the guppy (*P. reticulata*). Blanckenhorn & Hellriegel (2002) demonstrated sperm length to increase with increasing developmental temperatures from 12°C to 18°C and then to decrease as developmental temperature increased to 24°C. A replicate within the same study showed sperm length to increase with larval developmental temperatures from 15°C to 23°C. Thus, although Blanckenhorn & Hellriegel (2002) demonstrated phenotypic plasticity in sperm size, there was large variation in sperm size across 'replicates', plus the response of sperm size to temperature was different across replicates. Kindlemann *et al.* (2001) proposed that variation in developmental temperature may result in variation in adult mortality levels, that in turn is based on variation in the allocation of metabolic energy during ontogeny, such that those larvae that survive allocate resources away from reproduction to maintenance. Kindleman *et al.* (2001) also suggested that the observed reduction in the size of adults reared as larvae at higher temperatures may result from an increase in the rate

of assimilation (metabolic energy) and an increase in the conversion rate of gonadal biomass coupled with elevated levels of senescence. Thus, food quality is likely to alter assimilation but not conversion or senescence, whereas increase in temperature will increase all three parameters, resulting in smaller adults. In some ecothermic organisms (e.g. the Atlantic silverside, *Menidia menidia* (L.)) rapid growth is observed via thermal specialization resulting in physiological changes that determine the assimilation of (metabolic) energy resources favouring developmental reaction norms that result in trade-offs between growth rate and gamete size (Yamahira & Conover, 2002).

Given the repeated effect of developmental temperature on primary reproductive traits; e.g. testes size (Hellriegel & Blanckenhorn, 2002) and in particular egg size (Azevedo *et al.*, 1996; Fischer *et al.*, 2003b), it is surprising that only three studies have examined the effect of developmental temperature on sperm size. Therefore, I examine the effect of larval rearing and post-eclosion temperature on the expression of sperm size in *C. maculatus*. Alteration of the environment during development (larval rearing) is likely to alter the biochemical and metabolic processes that impact on the timing of synthesis of hormones that are crucial to phenotypic expression (Partridge & French, 1996; Nijhout & Emlen, 1998; Nylin & Gotthard, 1998; Nijhout, 2003b; Davidowitz & Nijhout, 2004; Angilletta, 2009), however alteration of temperatures after eclosing as adults (post-eclosion temperature) may not have any effect on the underlying biochemical processes and the subsequent expression of phenotypes. Thus, the expectation is that developmental temperature will affect sperm size.

3.2 Materials and Methods

3.2.1 The effect of larval rearing temperature on the adult phenotype

Approximately 1000 adults (estimated by mass) were housed with 200g of moth beans (~ 7500 beans) for one hour at 27°C, 32% RH. The egg-laden beans were then separated into four triple vent 110 mm Petri dishes (Fisherbrand, www.fisher.co.uk), with approximately 1500 beans per Petri dish. These were placed into 3 separate incubators (Lucky Reptile Herp Nursery II, The reptile shop, UK) at 17°C, 25°C and 33°C, with the 4th Petri-dish being incubated at 27°C, 32% RH within the insectary. Prior to adult emergence, the beans were plated out into individual cells of a 25 cell replidish at one bean per cell and sealed with a glass lid. As adults emerged from the seeds they were isolated within the cells, ensuring virginity and kept in their respective environments until they were 24 – 48h old. The emergent adult males and females were paired and allowed to copulate in the insectary at 27°C, males and females were paired within their treatments such that 17°C males were paired with 17°C females and so forth. A sub-set of these beetles were placed in a freezer -5°C immediately after copulation, for subsequent dissection to determine the number of sperm transferred, testis size and sperm length. After copulation was complete, females were kept individually in 110mm diameter Petri dishes with 40 fresh moth beans for oviposition, which were replaced daily with fresh moth beans every 24h (30 beans on day 2, 20 beans on day 3, 10 beans on day 4 and 10 beans on day 5 until death) in order to record daily egg laying rate and egg size. Males were kept separately in a Petri dish until death.

The number of sperm transferred at copulation was estimated by

dissecting the male spermatophore from the females' bursa copulatrix. Using watchmaker's forceps, the spermatophore was transferred into 100µl of insect saline mixed with biological liquid detergent until the spermatophore dissipated, (approximately 5 minutes). This solution was then stirred for 60 seconds before pipetting 20µl onto a haemocytometer (Marienfeld, Neubauer improved, two counting nets). Sperm were counted using a light microscope, from each of the five large squares (four corner squares and a middle square). Three haemocytometer measures per sample were taken.

Testis size was determined by dissecting defrosted males under an Olympus dissecting microscope [SZH10] linked to an Image analysis workstation (Moticam 2000). Each testis is approximately spherical (Gay *et al.*, 2009) and is made up of a large and small element with a narrow waist separating the sections (Gill *et al.*, 1971). Testes were dissected free from connective and fatty tissue and the 2D cross sectional area measured using Moticam 2000 image analysis software. To measure sperm length, the testes were transferred onto a microscope slide with a drop of insect saline and gently ruptured using fine bodied forceps. A cover slip was then placed over the slide and sperm viewed under dark-field (10X) using Nikon Eclipse E600 microscope. Images of the sperm were captured using Moticam 2000 and measurements taken using the segmented hand tool of Image J. Egg dimensions (10 eggs from each day) were measured directly from the surface of the bean. Length and width (mm) were measured under an Olympus dissecting microscope [SZH10] linked to Moticam 2000 image analysis work station.

A full replicate of the effect of larval rearing temperature on sperm

size, sperm number and testes size was carried out.

3.2.2 The effect of post-eclosion temperature on primary reproductive traits

Egg-to-adult development took place in the insectary at 27°C and the effects of post-eclosion temperature were studied on adults that had spent 24 – 48h post eclosion at either 17°C, 25°C, 27°C or 33°C. Adults between 0 – 2h post eclosion derived from larvae reared at 27°C were randomly distributed to the four different temperature environments for 24 – 48h. After 24 – 48h at their respective temperature, male and female were paired and copulation duration recorded at either 17°C, 25°C, 27°C and 33°C. A sub-set of mated beetles from the four temperature treatments were placed in a freezer at -5°C immediately after copulation, for subsequent dissection to determine the number of sperm transferred, testis size and sperm length as detailed above. After copulation was complete, females were isolated in individual Petri dishes and given 40 fresh moth beans on which to oviposit and every 24h females were provided with fresh beans so as to record daily egg laying rate and egg size. Males were kept separately in a Petri dish until death. The parametric data presented here was analyzed for both deviations from normal distributions and for heterogeneous variances between the various treatment groups. All traits were analysed using one-way ANOVA on SPSS version 20 (IBM) and significant differences between groups were established using *Post-hoc* tukey tests.

To investigate differences in traits as a result of different developing conditions, I employed analyses of variance. Given that body size is allometrically related to most traits, these analyses require control for the

effect of size on these traits. Residuals from ordinary least-square regressions (OLS) of body size on studied variables are often obtained to produce size-effect-free variables (Green, 2001). However, it has been shown that multiple relevant assumptions may not hold for residuals, and that the residual index is an ad-hoc sequential procedure with no demonstrated statistical justification (Garcia-Berthou, 2001; Green, 2001). This problem is solved with the use of analyses of covariance (ANCOVA), where the allometric influence of body size is controlled by treating it as a covariate.

3.3 Results

Larval rearing temperature affected sperm length, with males reared at 27ºC producing the longest sperm in both replicate 1 (ANOVA: $F_{3,36} = 9.160$, $P < 0.0001$) and replicate 2 (ANOVA: $F_{3,36} = 9.964$, $P < 0.0001$, Fig. 10). A two-way ANOVA with larval rearing temperature (fixed) and replicate (random) as factors revealed a statistically significant effect of larval rearing temperature on sperm length (ANOVA: $F_{3,71} = 15.6$, $P = 0.025$) and statistically non-significant for replicate (ANOVA: $F_{1,71} = 0.11$, $P = 0.754$). Post-eclosion temperature (all larvae reared at 27°C) had no effect on sperm length (ANOVA: $F_{3,36} = 0.57$, $P = 0.638$). Sperm length (μm) at 17°C = 0.171 ± 0.001, 25°C = 0.172 ± 0.001, 27°C = 0.169 ± 0.002 and 33°C = 0.171 ± 0.001.

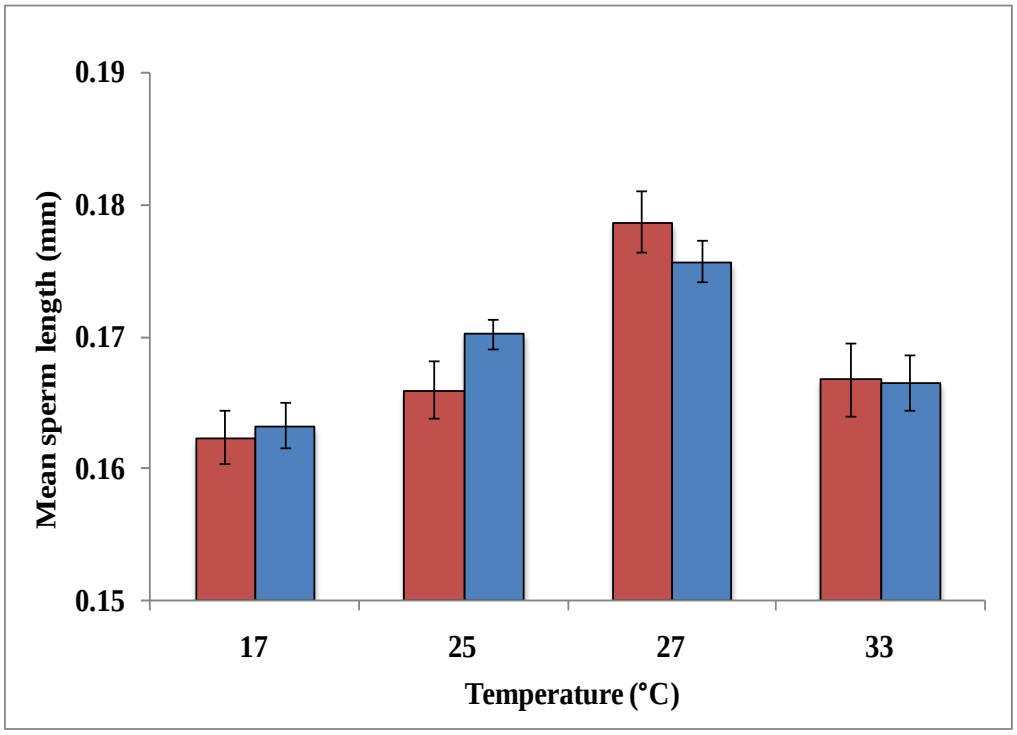


Fig. 10 Mean (±) standard error sperm length with regard to larval rearing temperature. Red bars represent replicate 1 and blue bars represent replicate 2.

Absolute testes size declined with increasing larval rearing temperature (replicate 1, ANOVA: $F_{3,35} = 3.37$, P = 0.03 and replicate 2, ANOVA: $F_{3,36} = 5.24$, P = 0.04, Fig. 11). A two-way ANOVA with larval rearing temperature (fixed factor) and replicate (random factor) revealed a statistically significant effect for larval rearing temperature (ANOVA: $F_{3,68} = 21.8$, P = 0.01) and statistically non-significant effect for replicate (ANOVA: $F_{1,68} = 8.80$, P = 0.058). Post-eclosion temperature (all beetles reared at 27°C) had no effect on absolute testes size (ANOVA $F_{3,36}=1.897$, P = 0.148): 17°C = 0.190 mm^2 ± 0.007, 25°C = 0.179 mm^2 ± 0.106, 27°C = 0.170 mm^2 ± 0.005 and 33°C = 0.164 mm^2 ± 0.008.

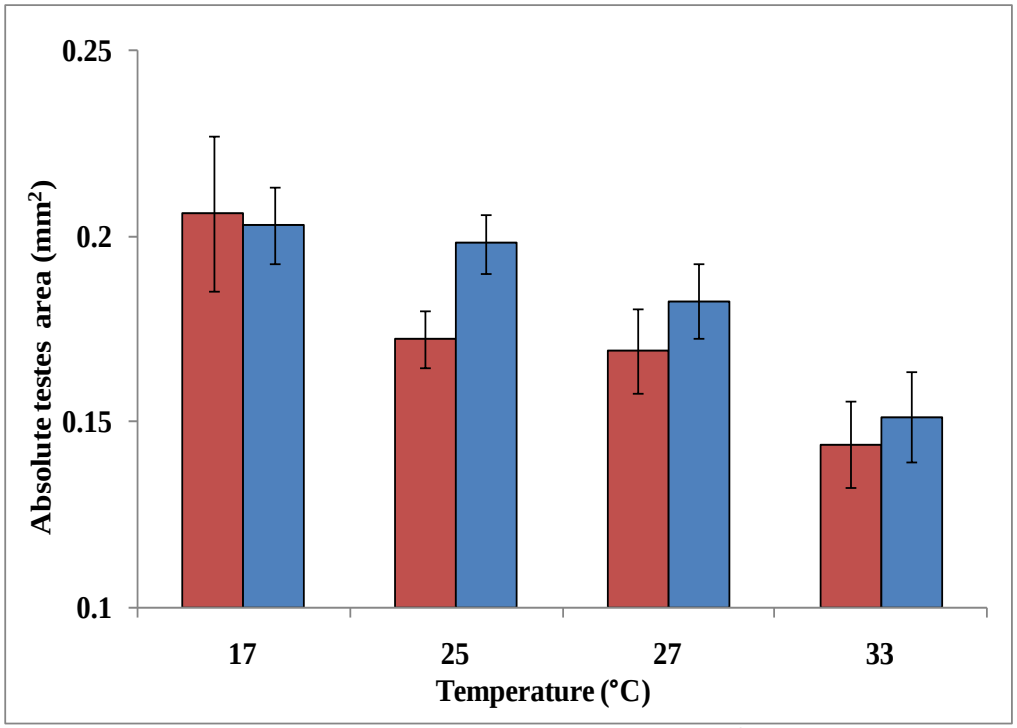


Fig. 11 Mean (±) standard error absolute testes size (mm^2) in relation to larval rearing temperature. Blue and red bars denote replicate 1 and 2 respectively.

Since larval rearing environment also influenced body size (elytra length, see chapter 2); larvae reared at 17°C eclosed to be bigger adults than larvae reared at 25°C, 27°C or 33°C ANOVA: ($F_{3,160}$ = 24.4, P < 0.0001). Thus, testes size was analysed in a model with body size included as a covariate; ANCOVA revealed no effect of larval rearing on relative testes size: replicate 1 (ANCOVA: $F_{3,30}$ = 1.10, P = 0.36) and replicate 2 (ANCOVA: $F_{3,30}$ = 1.16, P = 0.34).

Larval rearing temperature had a significant effect on the number of sperm transferred to the female (replicate 1, ANOVA $F_{3,61}$ = 4.74, P = 0.005; replicate 2, ANOVA: $F_{3,36}$ = 4.64, P = 0.008, Fig. 12) with adults reared as larvae at 25°C transferring the greatest number of sperm and those reared at 17°C transferring the fewest (Fig. 12). A two-way ANOVA with larval rearing temperature (fixed factor) and replicate (random factor) revealed a significant

effect of developmental temperature and a non-significant effect of replicate on the number of sperm transferred (ANOVA $F_{3,100} = 7.90$, $P < 0.0001$ and ANOVA $F_{1,100} = 1.70$, $P = 0.19$) respectively. Post-eclosion temperature had no effect on the number of sperm transferred (ANOVA $F_{3,58} = 0.43$, $P = 0.73$): 17°C = 68,750 ± 6,752, 25°C = 77,666 ± 7,327, 27°C = 72,812 ± 6,089 and 33°C = 67,777 ± 6,919.

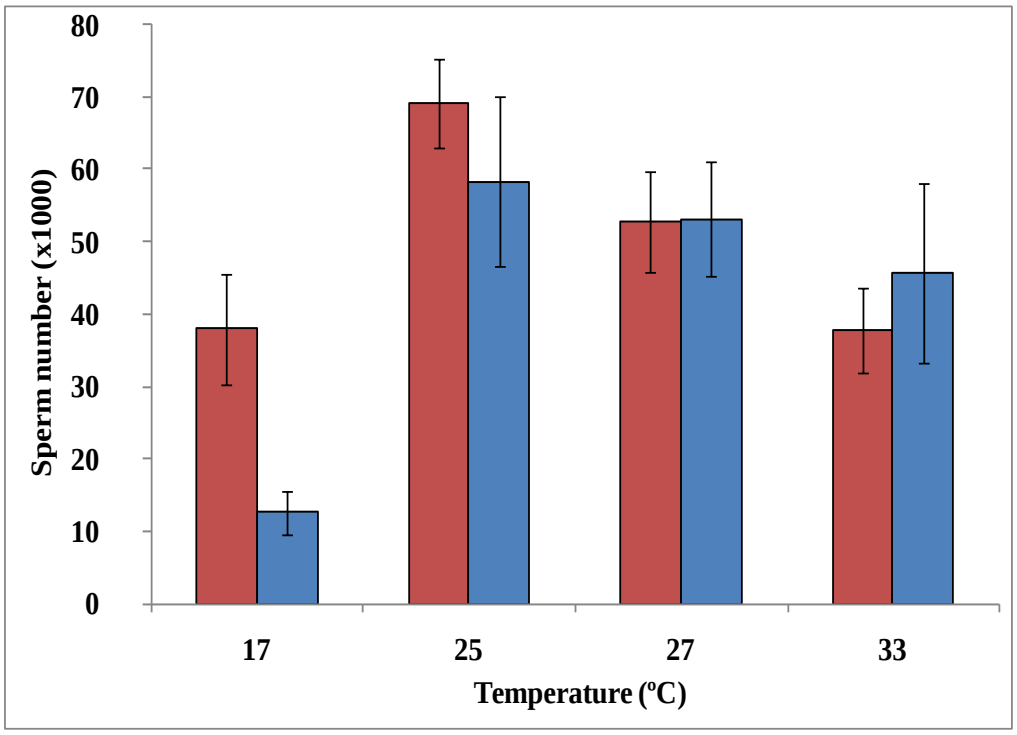


Fig. 12 Mean (±) standard error sperm number transferred in relation to larval rearing temperature. Blue and red bars denote replicate 1 and 2 respectively.

Females reared as larvae at 17°C laid fewer eggs than females reared under the other rearing temperatures (ANOVA: $F_{3,77} = 12.00$, $P < 0.0001$, Fig. 13), despite the fact that these females were largest. Female fecundity was also influenced by post-eclosion temperature, with life-time female fecundity lowest at 17°C (ANOVA $F_{3,56} = 10.19$, $P < 0.0001$, Fig. 14).

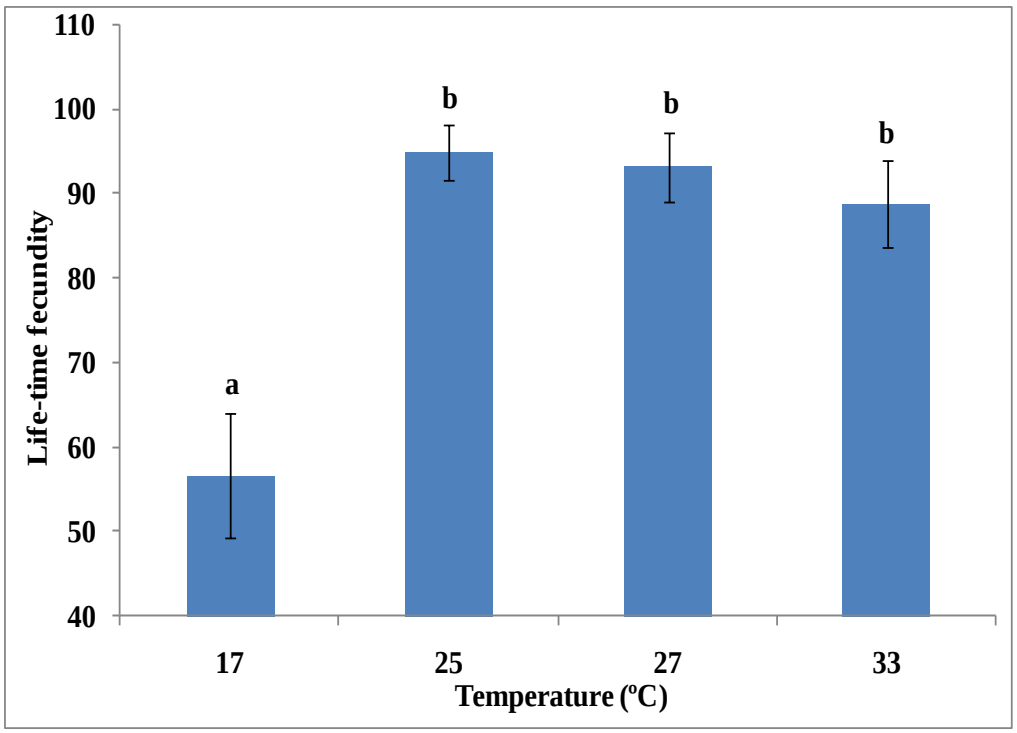


Fig. 13 Mean (±) standard error life-time fecundity of adults reared as larvae at different temperatures. Different superscripts indicate groups that were significantly different as revealed by a *Post-hoc* tukey test.

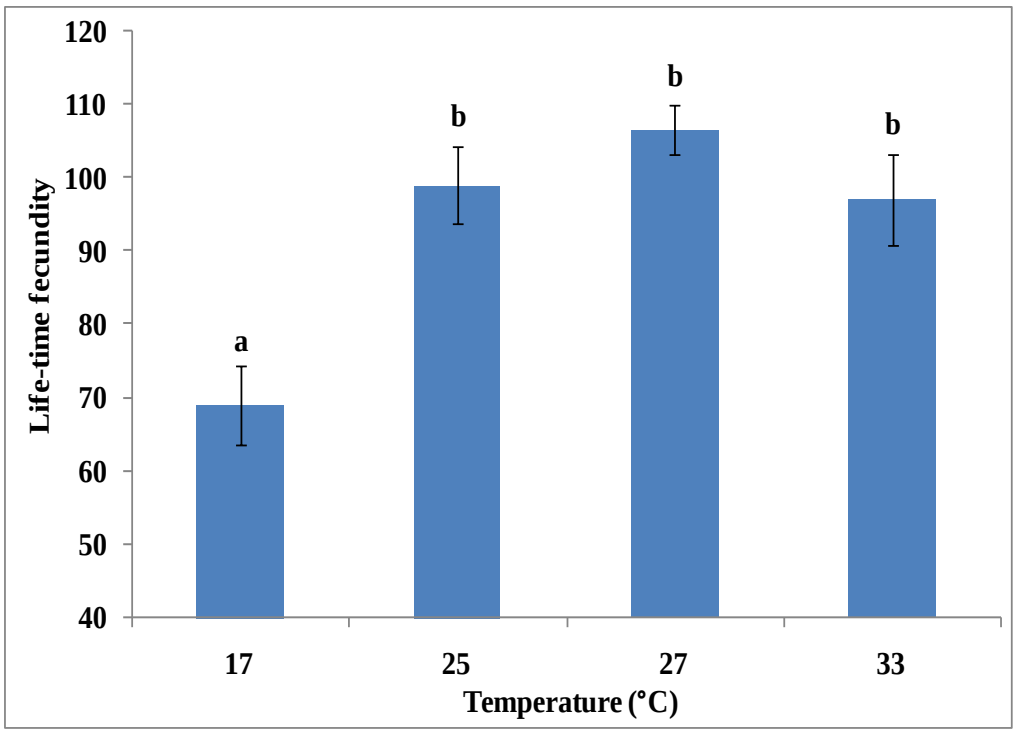


Fig. 14 Mean (±) standard error life-time fecundity of adults at different post-eclosion environments. Different superscripts indicate groups that were significantly different as revealed by a *Post-hoc* tukey test.

Larval rearing temperature also affected egg size. Egg length and width was greatest at 17 and 33°C. Egg length (ANOVA $F_{3,374} = 17.79$, $P < 0.0001$, Fig. 15), egg width (ANOVA $F_{3,374} = 15.858$, $P < 0.0001$, Fig. 15). Post-eclosion temperature also influenced egg size. Mean egg length was bigger at the extremes, 17°C and 33°C (ANOVA $F_{3,282} = 11.615$, $P < 0.0001$, Fig. 16) and mean egg width at 17°C (ANOVA $F_{3,282} = 18.894$, $P < 0.0001$, Fig. 16).

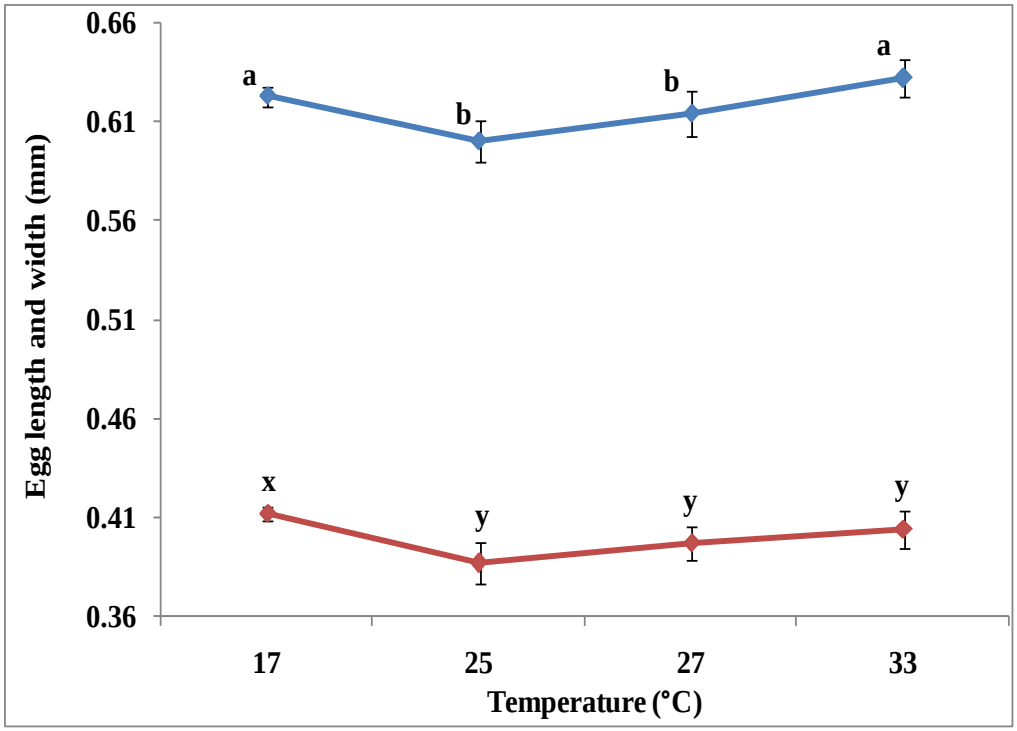


Fig. 15 Mean (±) standard error egg length (blue line) and egg width (red line) when larvae were reared at different temperatures. Different superscripts indicate groups that were significantly different as revealed by a *Post-hoc* tukey test.

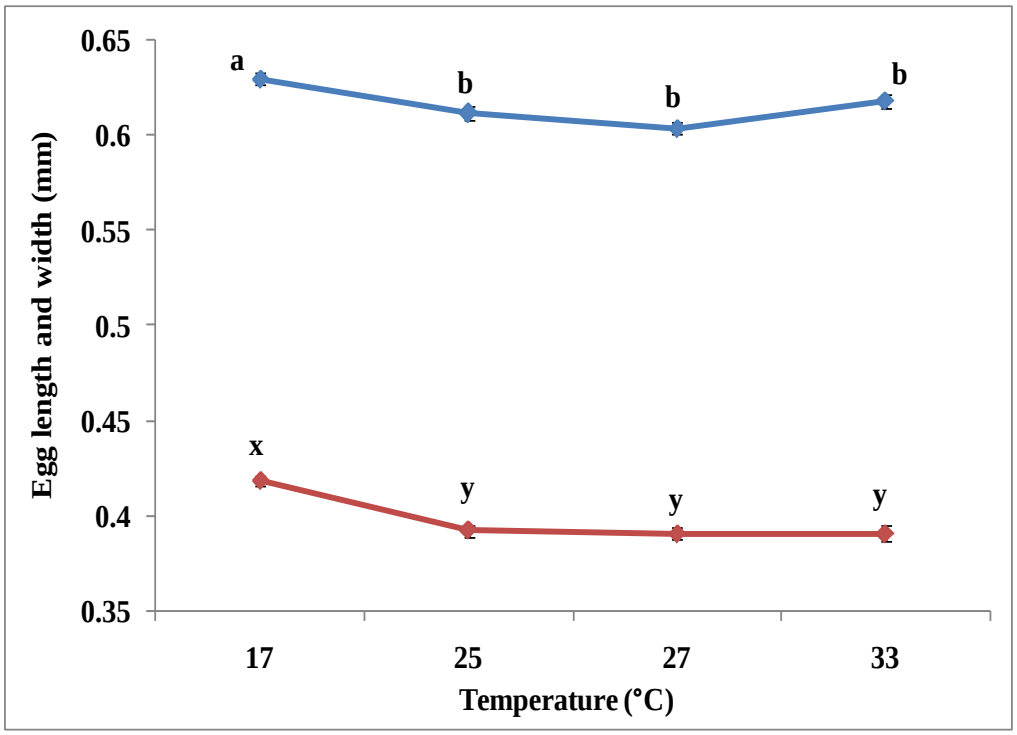


Fig. 16 Mean (±) standard error egg length (blue line) and egg width (red line) at post-eclosion temperatures. Different superscripts indicate groups that were significantly different as revealed by a *Post-hoc* tukey test.

3.4 Discussion

This is one of only a handful of studies (Blanckenhorn & Hellriegel, 2002; Vermeulen *et al.*, 2009; Immler *et al.*, 2010; Breckels & Neff, 2013; Minoretti *et al.*, 2013) to show phenotypic plasticity in sperm size and the 4th ever study to report sperm length to be affected by developmental rearing temperature; with sperm length being shortest at the two temperature extremes, 17°C & 33°C. The observed differences in sperm length are unlikely to be a consequence of difference in testes size as males reared at 17°C had the largest absolute testes, whilst those reared at 33°C had the smallest absolute testes. Blanckenhorn & Hellriegel's (2002) study of sperm length in *S. stercoraria* was the first ever study to report an effect of larval rearing temperature on sperm size. In some respects they found similar results to those presented here, with males derived from larvae reared at high and low temperature extremes producing the smallest sperm in one of their replicates. Minoretti *et al.* (2013) showed that in the land snail (*A. arbustorum*), developmental temperature explained about 24% of the variability in sperm length with snails reared at high temperatures (20°C) producing shorter sperm in comparison to offspring reared at 11°C and 15°C. Similarly, in the guppy (*P. reticulata*), Breckles & Neff (2013) showed individuals exposed to high temperatures during development (30°C) produced shorter sperm in comparison to individuals exposed to 23°C, 25°C or 28°C.

An intriguing question to arise from these observations is how does developmental temperature affect sperm size? Bergmann's rule generally extends to cells (Blanckenhorn & Hellriegel, 2002; Hellriegel & Blanckenhorn, 2002; Nijhout, 2003b), including eggs (Azevedo *et al.*, 1996;

Blanckenhorn, 2000; Fischer *et al.*, 2003a,b). One argument for larger cells at cooler temperatures is that energy assimilation (e.g. nutrient consumption and absorption) is less affected by temperature than energy dissimilation (metabolism). This would result in relatively less energy available for growth at higher temperatures, resulting in smaller cells and smaller individuals at higher temperatures (Nijhout, 2003b; Edgar, 2006). Another possible explanation is based on oxygen distribution; oxygen diffusion depends only weakly on temperature, whereas oxygen consumption depends strongly on it. Thus, cell size is predicted to be smaller at higher temperatures (Woods, 1999). However, these may explain why cells and bodies are larger at lower temperatures, but it does not explain why sperm size is smaller at lower temperatures.

One potential explanation could be that cell size appears to be at least partially under the control of insulin in *M. sexta* (Nijhout, 2003b; Edgar, 2006). Over expression of the *Drosophila* insulin-like proteins causes an increase in body size which is associated with an increase in both cell size and cell number (Partridge *et al.*, 1994; Nijhout, 2003b). Loss of function of insulin receptors results in a decrease in body size in *M. sexta* (Nijhout, 2003b). Thus, it is possible that temperature affects the release of these hormones which then affects body size, organ size and cell size across ectothermic species. However, this still does not explain why sperm cells are affected differently, although in insects, the growth of one tissue is altered by the presence or absence of another tissue. Thus, during growth the internal organs appear to be in competition for resources (Nijhout, 2003b). Therefore, the increased growth of other body organs at low temperatures may divert

resources away from sperm cell growth. However, this is purely speculative, although it does fit with the general concept that under stress resources get diverted to somatic maintenance and away from reproduction (Nijhout, 2003b).

Many insect species undergo compounded growth, i.e. growth that involves complex processes of the neuroendocrine system that controls the timing of the synthesis of hormones by regulating hormonal pathways (Nijhout, 2003a,b). This effects the duration of the larval stages and larval moulting is affected (Nijhout, 2003a,b). The factors involved in spermatogenesis, such as increased plasma titres of steroids like testosterone and other metabolites, can be altered at higher temperatures, resulting in changes to the timing and production of sperm under extreme stressful environments (Olsson *et al.*, 1997). For example, in reptiles elevated levels of testosterone accelerate metabolic processes pertaining to reproductive investment (see Gupta & Thapliyal, 1985). If gamete formation is a thermally sensitive process, then under normal growth conditions optimal gamete size will result without any shift in metabolic energies between cell growth and cell division. However, where metabolic energy is diverted towards maintaining elevated growth rates at extreme temperatures, cellular growth of gametes may be affected (Angilletta *et al.*, 2004a,b; Angilletta, 2009).

Plasticity of traits may be explained in terms of physiological and biochemical properties of the lipid composition in biological membranes. Van Dooremalen *et al.* (2011) found the fatty acid composition of membrane and storage lipids of the springtail (*Orchesella cincta*), to exhibit phenotypic plasticity in response to high temperatures. This is thought to be adaptive in

that changes in the lipid content of membranes regulate cell membrane permeability and thus cellular metabolism, in the face of heterogeneous thermal environments. Alterations to cell membrane permeability are also likely to affect the movements of genes product, including hormones that are important to developmental regulation. The timing of the production of these hormones during development could affect the subsequent patterns of growth of organs, including testes. At a mechanistic level, a number of candidates could produce the observed plasticity in sperm size by inducing changes to protein production and thus the regulatory processes associated with the homeostasis of cellular signalling (*transcriptome*) that underlie the biochemical and hormonal metabolism (*proteome*), thereby bringing about changes at the level of the phenotype (Schlichting & Smith, 2002).

Alteration of sperm size in response to developmental temperature is likely to alter the functional characteristics of the sperm and ultimately male fertilization success, especially in the face of sperm competition. Sperm motility and longevity are likely to be related to sperm size (Snook, 2005). A longer duration of sperm motility has been shown to increase fertilization success in the myobatrachid frog (*Crinia georgiana*) (Dziminski *et al.*, 2009), whilst in the guppy (*P. reticulata*), fertilization success was positively related to sperm number and sperm swimming velocity (Boschetto *et al.*, 2011). Similar results have been reported for Walleye (*Sander vitreus*), and the green swordtail (*Xiphophorus helleri*) (Casselman *et al.*, 2006; Gasparini *et al.*, 2010). Pattarini *et al.* (2006) showed that variability in sperm length in *D. melanogaster* was closely related to male success during sperm competition. They found that males with bigger sperm (longer flagella length) had greater

fertilization success.

In *D. melanogaster*, Morrow *et al.* (2008) found additive genetic variation in sperm length and a small, but statistically significant, environmental effect as a result of larval rearing density. Morrow *et al.* (2008) concluded that genotype x environment interactions were responsible for the maintenance of standing genetic variation in sperm size between males. Although, Morrow *et al.* (2008) demonstrated phenotypic plasticity in sperm length, they suggested this trait was *canalized*, resistant to any change in morphology as a result of variation in developmental conditions; as a result of no obvious support for the condition-dependent expression of sperm length.

In the present study, smaller males (from 33°C) and larger males (from 17°C) had similar sized sperm despite their absolute testes size being significantly different. Vermeulen *et al.* (2009) showed that in the scorpionfly (*P. vulgaris*), males of the first summer generation that were reared at higher densities and fed *ad lib* grew into larger males that produced shorter sperm in comparison to males that were kept singly, with reduced food availability. However, because density and food availability were confounded it is not clear within the study of Vermeulen *et al.* (2009) whether larval rearing density or food deprivation brought about the observed plasticity of sperm size. In the Indian meal moth (*Plodia interpunctella*), larval food deprivation had no effect on sperm length (Gage & Cook, 1994).

A few studies have reported conditions during larval development to affect male testes size and sperm number. Gage (1995) found male Indian meal moths reared at high densities invested more in testes and consequently produced greater sperm numbers than those reared at low densities. Hellriegel

& Blanckenhorn (2002) found testes size to increase with decreasing developmental temperature in the yellow dung fly (*S. stercoraria*), in line with the general trend of increasing organ size at lower developmental temperatures (Nijhout, 2003b). In chapter 2, males reared at high temperatures (33°C) reached adult eclosion quicker than those reared at 17°C. Thus development at 33°C was faster in comparison with 17°C males: resulting in smaller adults at 33°C that also had smaller testes (see also Nijhout, 2003b).

The effect of post-eclosion temperature on sperm number shown in this study appears to contradict Katsuki & Miyatake (2009) who found copulation in the bruchid beetle *Callosobruchus chinensis*, low ambient temperatures resulted in an increase in copula duration and a corresponding increase in the number of sperm inseminated. In the present study, post-eclosion temperature had no effect on the number of sperm transferred during copulation across the different thermal environments. However, Katsuki & Miyatake (2009) measured sperm number from the spermathecae which is not the primary site of sperm deposition in bruchids (Eady, 1994). Thus, it is possible that Katsuki & Miyatake (2009) may have incorrectly measured the number of sperm transferred.

In the present study egg size was greatest in females reared at the two temperature extremes (17°C and 33°C). In dung flies (*S. stercoraria*) and (*S. cynipsea*), low developmental temperatures at high altitudes (1500m) resulted in the production of bigger eggs (Blanckenhorn, 1997). This is thought to lower the risk of desiccation and/or provide sufficient resources for larval development under stressful conditions (Blanckenhorn, 1997). In the tropical butterfly (*Bicyclus anynana*), females that oviposited at low temperature (post-

eclosion) produced fewer but larger eggs (Fischer *et al.*, 2003a,b). A similar pattern was found in the present study: fecundity was shown to increase with rearing temperature and egg size increased at the temperature extremes (17°C and 33°C). Similar egg size plasticity has been documented as a result of altering developmental temperature in *D. melanogaster* (Azevedo *et al*., 1996; Sheader, 1996).

Larger eggs at lower temperatures could provide offspring with more resources and thus a greater possibility of survival. In *B. anynana,* larger eggs (those laid at 20°C) attained a greater hatching success (at 27°C) and larvae from 20°C attained higher probability of reaching maturity in comparison to those larvae reared at 27°C when the eggs were switched to 27°C. Larger egg size at low temperatures could be due to: i) vitellogenesis: a change in the production of oocytes in relation to oocyte growth, that affects both the size and number of eggs produced by the female (van der Have & de Jong, 1996); and/or ii) larger body size at low temperature enabling the production of bigger eggs (Azevedo *et al.*, 1996; Fischer *et al.*, 2003b; Steigenga, 2008; Liefting *et al.*, 2010).

The nature and magnitude of life-history traits in response to environmental variation is primary to the evolutionary process. Variation during development (plasticity) can contribute to changes in the trait reprtoire and ultimately bring about genetic diversification between populations (Moczek, 2010). Brakefield (2006) and Shubin *et al.* (2009) suggest genes "behave as followers" to environmental cues and that much of the observed morphological diversity is old genes performing new tricks (West-Eberhard, 2005a,b). However, evolution may be constrained by developmental

mechanisms that are also underpinned by developmental temperature (Zera & Harshman, 2001; Price *et al.*, 2003; Crispo, 2008). Developmental temperature drives both growth and growth regulating biochemical mechanisms (Nijhout & Emlen, 1998; Zera *et al*., 1998; Zera & Harshman, 2001), thus, thermal fluctuations may affect the gene expression of individual cells and hence the final size of the cells (Pétavy *et al.*, 2004).

In conclusion, the external environment plays an important role during larval development in determining the rate of growth and development. However, the exact developmental pathways, biochemical mechanisms and/or the hormonal changes that bring about phenotypic plasticity are still unknown. In order to more fully understand how temperature affects the expression of sperm length it is useful to identify when, during development, these traits are sensitive to thermal conditions. This is the aim of the next chapter.

4. Thermal switch experiments reveal when during development, sperm length is temperature sensitive

4.1 Introduction

The temperature experienced during development has been shown to have dramatic impacts on morphogenesis and cell differentiation in a number of taxa (Pieau, 1996; Valenzuela, 2001; Lin *et al.*, 2008). For instance, in some reptilian embryos, a change to the temperature experienced during development determines gonadal differentiation. This subsequently identifies that individual as a male or female, which in turn drives the phenotypically plastic response of numerous other sex-related traits, giving rise to sexual dimorphism in terms of size, shape and colour (Pieau & Dorizzi, 1981; Pieau, 1996; Shine, 2004). Thus, the term thermal switch within the context of the present study refers to a technique of changing the temperature at certain larval stages. These stages are sensitive to temperature fluctuations which could affect the subsequent expression of the adult phenotype through changes in the regulatory mechanisms (Garen *et al.*, 1977; Belote & Baker, 1982; Stevens & Bryant, 1986; Belote & Baker, 1987; Bhat & Babu, 1987; Brakefield *et al.*, 1996; Kitamoto, 2001).

Environmental sex determination (ESD) has been classified as either type I or type II, with type I further sub-divided into type IA and IB (Valenzuela, 2001). Type IA is the male-female pattern, in which there is a single transition zone where 100% females are produced from incubation temperatures above a particular temperature and 100% males below this incubation temperature (±1°C transitional range). Type IB describes the

opposite situation in which 100% males are produced above and 100% females produced below a transition temperature (Mitchell *et al.*, 2006). By contrast, type II sex determination has two transition zones, female-male-female, wherein, at the temperature extremes female offspring are produced, whilst males are produced at the intermediate temperatures. Mixed sex ratios can be observed in the clutches of both type I and type II species and occasionally intersex individuals are produced around the pivotal temperature (Mitchell *et al.*, 2006).

In many ectothermic species, a short thermosensitive period (TSP) occurs during embryogenesis wherein sex determination takes place (Pieau, 1996). A key tool in the identification of TSPs has been the temperature shift experiment. A temperature shift, in this context, is defined as an abrupt change in environmental temperature during development that is induced by shifting the developing organism to either higher or lower environmental temperatures (for example, see Valenzuela, 2001; Lin *et al.*, 2008). Temperature shift experiments allow the interplay between the developmental environment and embryogenesis to be studied (Valenzuela, 2001) and have been used extensively on reptilian embryos to gain an understanding of the thermosensitive stages of sex determination (Shine, 2004).

In reptilian species the number of shift experiments has accumulated over the years (Yntema & Mrosovsky, 1982; Bull, 1983, 1987; Deeming & Ferguson, 1989a, 1991; Janzen, 1995; Ciofi & Swingland, 1997; Valenzuela, 2001; Shine, 2004; Yao *et al.*, 2004; Booth, 2006; Lin *et al.*, 2008; Shoemaker & Crews, 2009). More recently, studies related to global warming have investigated the impact of thermal shifts on the macro-ecology of ectothermic

species (Neuwald & Valenzulea, 2011). Two kinds of temperature switch experiments have been employed to study thermosensitive phases during embryonic developmental: the 'switch-once' and the 'switch-twice' experiment. The distinction between the two is that in shift-once experiments the developing embryo is shifted once to either a lower or higher temperature whilst in shift-twice experiments the embryo is shifted twice: first, to a higher or lower temperature (known as an intermediate temperature) and then switched back to the original temperature. Switch-once experiments are useful in studying thermosensitive phase in species with short developmental periods, whilst switch-twice experiments reveal both the stage and duration of the thermosensitive period. The latter tend to be used in species with prolonged development, such as Alligators (*Alligator mississippinensis*), which have over 20 developmental stages during embryogenesis (Deeming *et al.*, 1988; Deeming & Ferguson, 1989a,b, 1991).

Eggs of the Amazonian freshwater turtle (*Podocnemis expansa*) incubated at 30.5°C produced males whilst those incubated at 34.5°C produced females. Shift-once and shift-twice experiments revealed a pivotal temperature of 32.6°C that produced an equal number of males and females. In the European pond turtle (*Emys orbicularis*), eggs incubated at 25°C and 30°C produced 100% male and 100% female offspring respectively (Pieu & Dorizzi, 1981). A series of thermal switches between 25°C and 30°C at different developmental stages (stage 8 to stage 26) revealed that until stage 16 the gonads were undifferentiated. The critical phase for 100% male gonad formation at 25°C was shown to be between stages 16 to 21 and for 100% female gonadal formation at 30°C was shown to be between stages 16 to 22.

However, a two-stage switch at these developmental stages resulted in mixed sex-ratios and intersex individuals, revealing stage 16 to be crucial for both male and female gonadal differentiation in this species (Pieu & Dorizzi, 1981).

Thermal switch experiments have revealed the TSP to occur at approximately the same morphological stage in reptiles (Bull, 1987). For example, in the Alligator (*A. mississippiensis*), the TSP occurs when ~ 23% of embryonic development is attained, irrespective of the ambient temperature (Deeming & Ferguson, 1989a; Lang & Andrews, 1994). Similarly, in turtles the TSP corresponds to approximately 15 – 25% of embryonic development, whilst in the leopard gecko (*Eublepharis macularius*), it occurs at ~ 30% of embryonic development (Bull, 1987). Histological studies also point to the TSP corresponding to the first stage of gonadal differentiation (see Pieau *et al.*, 1999). However, the mechanistic basis of how temperature interacts with the genome of the developing embryo to affect the sex of the offspring is still not completely resolved.

In some reptilian species, temperature dependent sex determination is thought to be steroid hormone dependent wherein the absence of estrogens results in male gonadogenesis, whilst their presence results in ovarian development (Lance & Bogart, 1992). Injection of estrogens into the eggs of the marine turtle (*Dermochelys coriacea*) during the TSP resulted in the feminization of gonads in genotypic males whilst injection of antiestrogens (e.g. tamoxifen) resulted in the masculinization of gonads (Desgraves *et al.*, 1993). Aromatase (an enzyme complex), which converts androgens to estrogens during the TSP is thought to maintain ovary structure during ovary differentiation (Pieau *et al.*, 1999). Additionally, several homologous genes in

mammals and reptiles (e.g. *AMH, SF1, SOX9, WT1* and *DAX1*) are known to be associated with aromatase and involved in gonadal formation and differentiation (Pieau *et al.,* 1999). Across organisms, differences in the regulation of the aromatase gene have been reported (e.g. Pieau *et al.,* 1999). This is thought to be regulated by developmental temperature which induces variation in the transcription of the aromatase gene itself, thereby resulting in variation in gonadal gene expression (Pieau *et al.,* 1999).

In comparison to reptiles, relatively few studies have examined the effects of temperature switches during development in insects. Many of the early studies were primarily carried out to study the effects of fluctuating temperature on gene expression and subsequent phenotypic expression (Foster & Suzuki, 1970; Garen *et al.,* 1977; Belote & Baker, 1982; Stevens & Bryant, 1986; Belote & Baker, 1987; Bhat & Babu, 1987; Kitamoto, 2001). Belote & Baker (1982) were amongst the first authors to study the effects of temperature switching on a particular regulatory locus, *the transformer-2 (tra-2),* on the sex determination pathways in *D. melanogaster*. The *tra-2* is a temperature sensitive allele which behaves as a loss-of-function mutation at the restrictive temperature of 29°C. The presence of a functional *tra-2* gene in chromosomally female individuals causes them to develop normally whilst, mutant versions of the *tra-2* gene resulted in chromosomally female individuals developing into male features. Belote & Baker (1982) suggested that female features (e.g. female genital structures) are expressed through the repression of male genital primordium. Temperature-shifts from 16°C to 29°C on developing female *Drosophila* revealed the TSP for reproductive trait expression to be between larval instar II to early-middle pupal period. Female

flies shifted from 16°C to 29°C (i.e. switched off the *tra-2* gene at 29°C) during the TSP, often expressed a set of under-developed male genitalic structures (e.g. penis) that were sometimes found in close association with partially-developed female genital structures (Belote & Baker, 1982). An interpretation of this is that at 16°C the female primordium is permitted to grow and develop. However, when switched to 29°C the female primordium then follow a male developmental trajectory. Additionally, Belote & Baker (1982) tested for the function of *tra-2* in the formation of secondary sexual traits. A shift from 29°C to 16°C 12 hours into pupation resulted in the formation of bristles with female morphology but the number of bristles was the same as that found in males (Belote & Baker, 1982). Thus, at pupation, the number of bristles is fixed but the thickness and morphology of the bristles is determined later in development.

Garen *et al.* (1977) showed that thermal shifting in *D. melanogaster* from 20°C to 29°C early during instar III resulted in the failure of larvae to pupate, with the final instars remaining as a free living larvae. This was associated with low circulating levels of ecdysone, necessary for larvae to enter pupation (Garen *et al.*, 1977). Thermal-shift experiments carried out after the onset of pupation were shown to be less effective than thermal shifts carried out earlier during development in altering body size in *D. melanogaster*. This is thought to occur because the imaginal disc that produces the wing and thorax is formed early during ontogeny, prior to pupation (French *et al.*, 1998).

In a study of the effects of developmental plasticity and adult acclimation to environmental variation, Brakefield *et al.* (1996) used thermal

switching (17°C to 23°C and vice versa) under laboratory conditions in the tropical butterfly (*Bicyclus anynana*). These temperatures mimicked the dry season (17°C) and wet season (23°C) polymorphism. Dry season adults have characteristically bigger bodies and smaller eye-spots on their wings (the cryptic form) whilst wet season adults have smaller bodies but larger wing eye spots. The dry season adults also have a lower metabolic rate and delayed reproduction. When wet season larvae were switched to dry season conditions (23°C to 17°C) during the final larval instar, the resultant adults were similar to those that developed under dry season conditions. When dry season larvae were switched to wet season conditions at the final instar (17°C to 23°C) the resultant adults were similar to those that developed under wet season. Thus, there appears to be a TSP somewhere around the final larval instar in this species. Brakefield *et al.* (1996) suggest that the environment during pre-imago development affects the timing and duration of ecdysone production and insulin signalling, which subsequently affects the adult phenotype in the tropical butterfly (*B. anynana*) (Brakefield *et al.*, 1996). To conclude, thermal switch experiments have been used extensively in developmental (ontogenic) studies to determine when during ontogeny developmental pathways are sensitive to temperature fluctuations.

In the previous chapter, it was found that males reared at the lowest (17°C) and highest (33°C) temperatures produced the smallest sperm. Here I use a switch-once experiment to discover when the expression of sperm size is thermo-sensitive. Developing larvae were switched from 17°C to 27°C and vice-versa and from 33°C to 27°C and vice-versa and the sperm length of the resultant adult males determined. To my knowledge this is the first study to

investigate the presence of a TSP that impacts on the phenotypic expression of sperm length.

4.2. Materials and Methods

Approximately 1000 adults (estimated by mass) were housed with 200g of moth beans (~ 7500 beans) for one hour at 27°C, 32% RH. The egg laden beans were then separated into 6 triple vent 110 mm Petri dishes (Fisherbrand, www.fisher.co.uk), with approximately 1500 beans per Petri dish. These were placed into 6 separate incubators (Lucky Reptile Herp Nursery II, The reptile shop, UK), two at 17°C, two at 27°C and two at 33°C.

Egg-laden beans were switched from 17°C to 27°C and vice-versa and 33°C to 27°C (and vice-versa) at differing times during larval development (Fig. 17 A–D). For each temperature a control set of egg-laden beans completed development at that temperature (i.e. no switch occurred). Prior to adult emergence, the beans containing the developing larvae were placed into individual cells of a 25 cell replidish and sealed with a glass lid. Petri dishes were checked for emergence every 24 hours. Upon eclosion approximately 20 males and 20 females (24 – 48h post-eclosion) were euthanized by freezing at -5°C in an eppendorf tube and stored. Mean elytra length of the 17°C to 27°C and the reciprocal 27°C to 17°C beetles were measured, whilst sperm length (mm) was measured in all thermal switch experiments (see below).

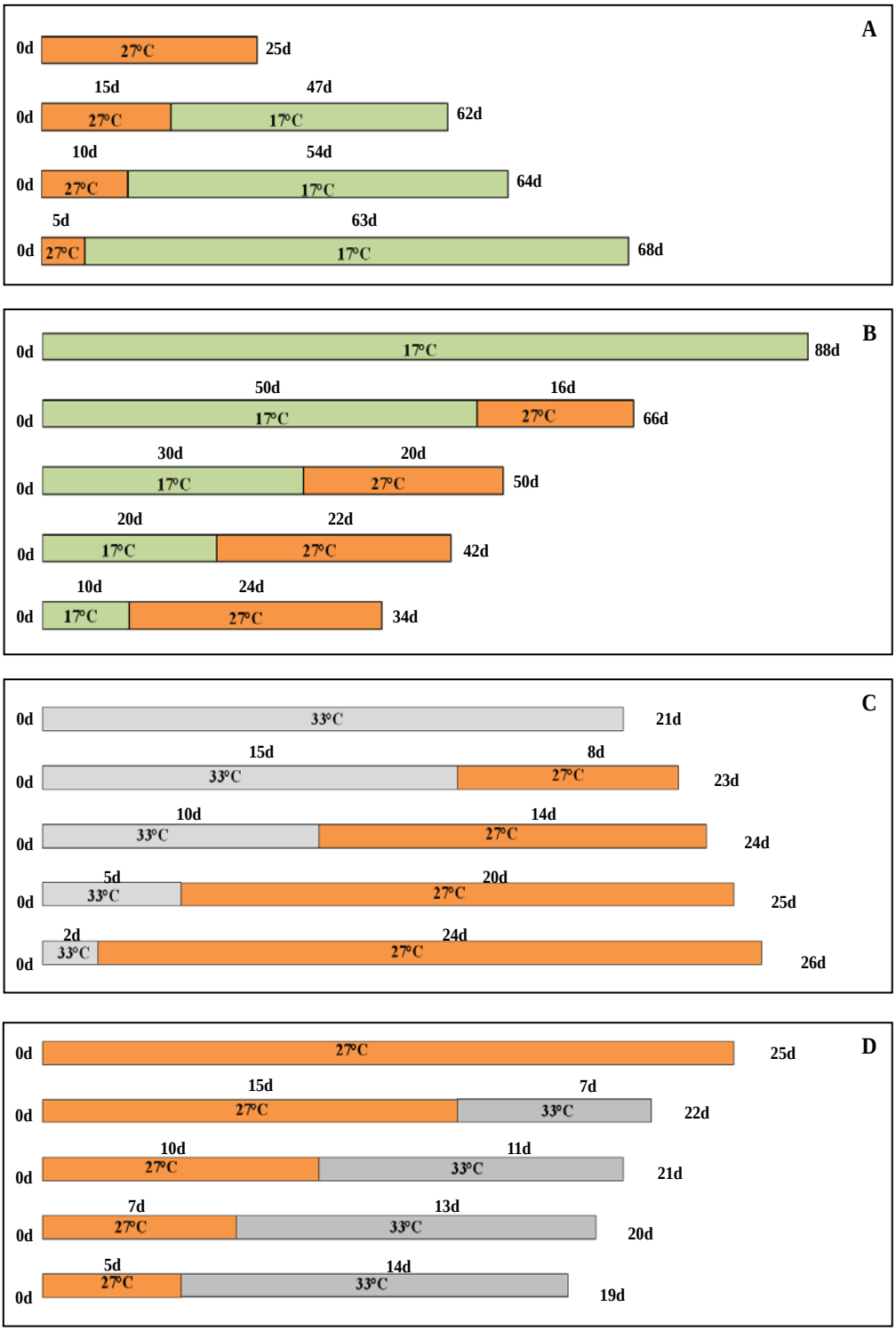


Fig. 17 (A–D) Visual representation of the thermal switch protocol A) larvae switched from 27°C to 17°C, B) larvae switched from 17°C to 27°C, C) larvae switched from 33°C to 27°C, D) larvae switched from 27°C to 33°C.

4.2.1. Measurements

Elytra lengths of twenty males and females were measured as in chapter 2. To measure sperm length, the testes were dissected out from males and transferred onto a microscope slide with a drop of insect saline where they were gently ruptured using fine bodied forceps. A cover slip was then placed over the slide and sperm viewed under dark-field (10X) using Nikon Eclipse E600 microscope. Images of the sperm were captured using Moticam 2000 and measurements were made using segmented hand tool of ImageJ after calibration. Ten to fifteen sperm per male (n = 18 – 22 males per treatment) were measured and a mean sperm size per male was determined. The parametric data presented here was analyzed for both deviations from normal distributions and for heterogeneous variances between the various treatment groups. All traits were analysed using one-way analysis of variance (ANOVA) on SPSS version 20 (IBM) and significant differences ($P<0.05$) of means between groups were established using *Post-hoc* tukey test.

4.3 Results

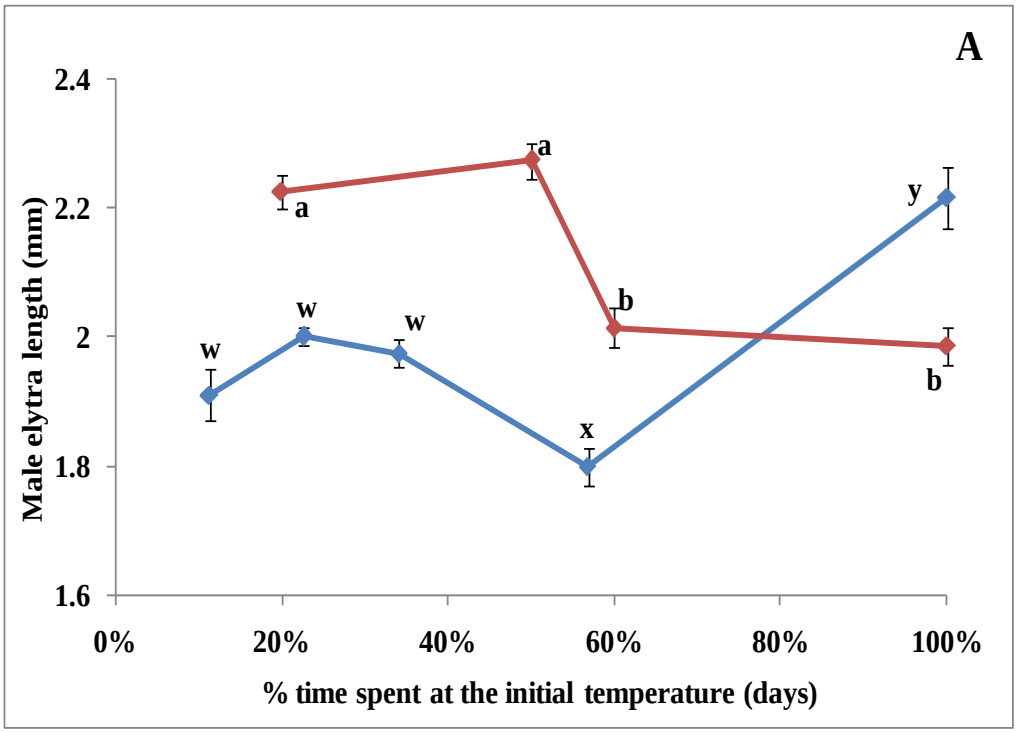
When larvae were switched from 17°C to 27°C up to day 50 of development (approximately 60% of development) their elytra lengths were comparable to those beetles that completed full development at 27°C. Thereafter, their elytra lengths increased and were of maximum length when incubated for the entire development at 17°C; males (ANOVA $F_{4,85} = 19.2$, $P < 0.0001$, Fig. 18A) and females (ANOVA $F_{4,82} = 13.1$, $P < 0.0001$, Fig. 18B). When developing larvae were switched from 27°C to 17°C within the first 10 days of development (approximately 50% of development) elytra lengths were comparable to those

beetles that completed full development at 17°C. Thereafter, elytra length decreased in size, males (ANOVA $F_{3,60} = 24.01$, $P < 0.0001$, Fig. 18A) and females (ANOVA $F_{3,57} = 5.1$, $P = 0.003$, Fig. 18B). Collectively, these results point to a TSP in the development of elytra length that occurs somewhere between 40% and 60% of development for the 27°C to 17°C switch and somewhere after 60% of development for the 17°C to 27°C switch.

Sperm length was longest when larvae experienced full development at 27°C mean = 0.172 ± 0.001 which is similar to replicate 1 and 2 from the results presented in chapter 3. Thermal switching from 27°C to 17°C within the first 10 days of larval development resulted in sperm that were relatively small (ANOVA: $F_{3,36} = 13.6$, $P < 0.0001$, Fig. 19A) and comparable in size to those that completed full development at 17°C. Sperm length in the reciprocal switch (17°C to 27°C) had smallest mean (0.160 ± 0.001) in the control group that were not switched (i.e. completed full development at 17°C) (ANOVA: $F_{4,45} = 19.8$, $P < 0.0001$, Fig. 19A). When males were switched from 17°C to 27°C within the first 50 days of development, sperm were relatively long and similar in length to those of males that completed their full development at 27°C. These data indicate that temperature experienced early during development has little or no effect on sperm size, with the TSP somewhere around 50% to 60% of development for the 27°C to 17°C switch or somewhere above 60% of development for the 17°C to 27°C switch.

For the 27°C to 33°C switch experiment, sperm length was greatest following full larval development at 27°C (mean = 0.174 ± 0.002 again confirming the results of chapter 3) and decreased as larvae spent more time developing at 33°C (ANOVA: $F_{4,47} = 15.3$, $P < 0.0001$, Fig. 19B). Full

development at 33°C resulted in a mean sperm length = 0.163 ± 0.001, very similar to those reported in chapter 3. Of interest, the TSP for the switch-up protocol (27°C to 33°C) appears to occur earlier during larval development, taking place somewhere between 30% to 40% of larval development. For the 33°C to 27°C switch, sperm length was greatest only when larvae experienced the first two days of development at 33°C. Thereafter, sperm length was reduced and remained constant (ANOVA: $F_{4,46} = 3.5$, $P < 0.014$, Fig. 19B), again suggesting a thermosensitive period relatively early in larval development.



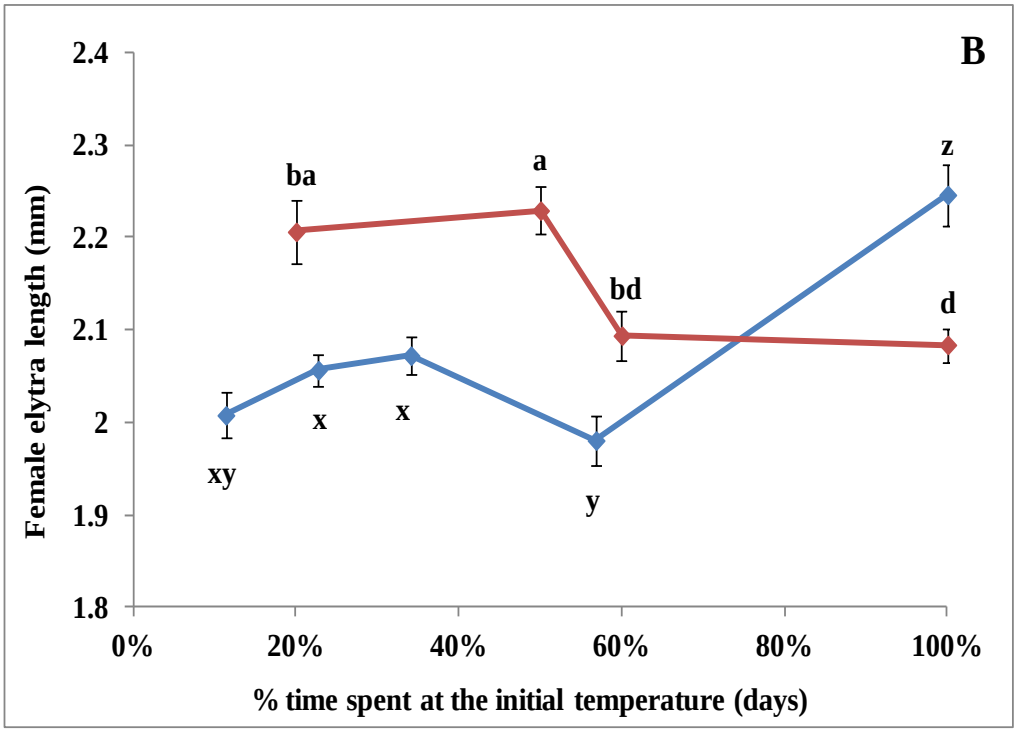


Fig. 18 (A–B) Mean (±) standard error elytra length (mm) of adult A) males and B) females, when larvae were reared at 17°C before being switched to 27°C (blue line) or 27°C before being switched to 17°C (red line). Different superscripts represent significant differences as revealed by *Post-hoc* Tukey tests ($P<0.05$), within switch protocols.

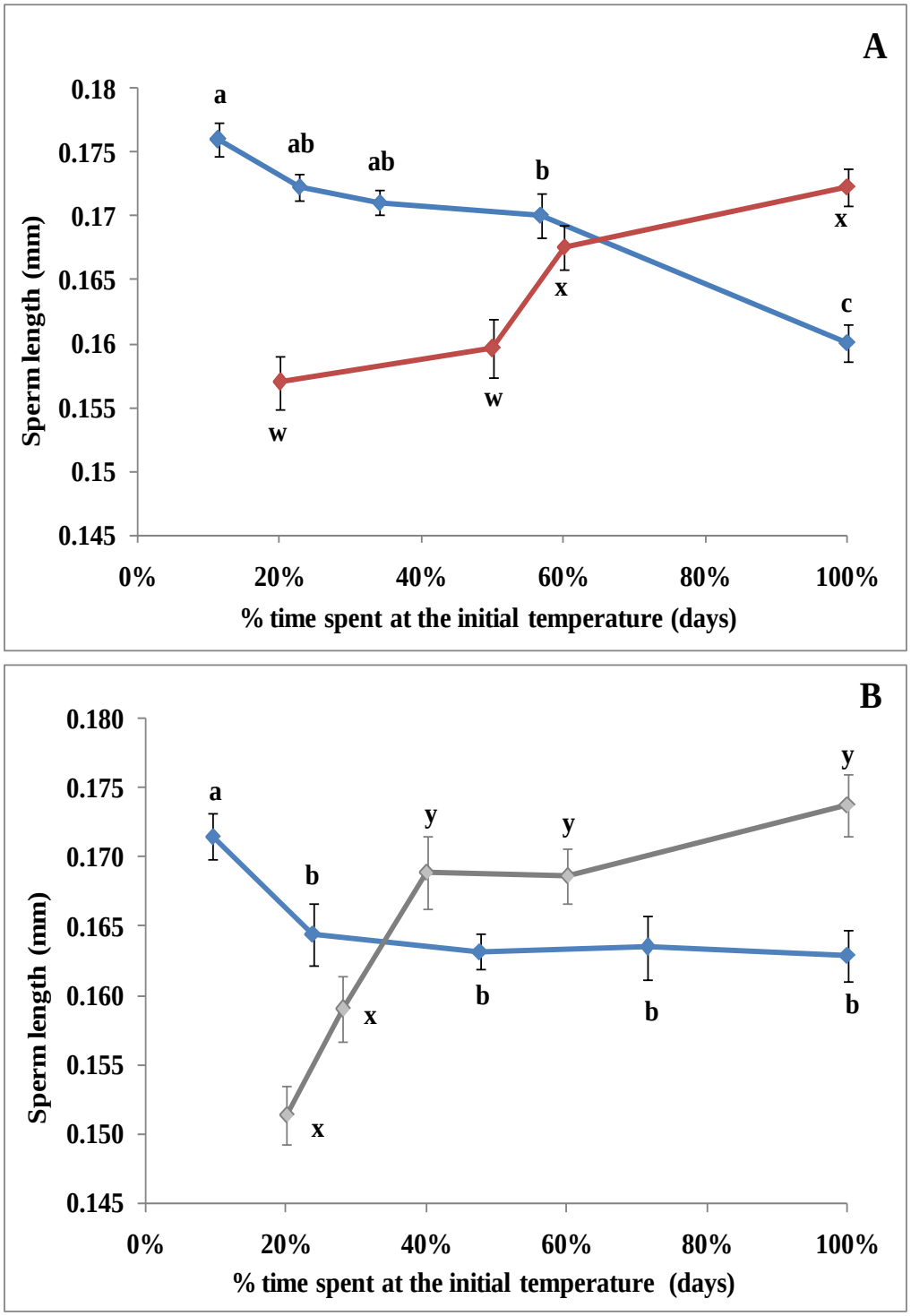


Fig. 19 (A–B) Mean (±) standard error sperm length (mm) in relation to % age time in development spent at different temperatures. In A) larval development at 27°C before being switched to 17°C (red line) or at 17°C before being switched to 27°C (blue line). In B) larval development took place at 27°C before being switched to 33°C (grey line) or, at 33°C before being switched to 27°C (blue line). Different superscripts represent significant differences within switch protocols as revealed by *Post-hoc* Tukey tests ($P<0.05$).

4.4 Discussion

This is the first study to apply a thermal switch protocol to investigate when, during development, the expression of sperm length is fixed. Switching the thermal regime during larval development altered the expression of both elytra length and sperm length. In the switch-down protocol (17°C to 27°C or 27°C to 17°C) the TSP for elytra length in both males and females occurred around 50% – 60% of development (27°C to17°C) or somewhere between 60% and 100% of larval development (17°C to 27°C). These times correspond to the developmental phase of instar III/ instar IV (chapter 2). A similar TSP for sperm length was found between 50% and 60% of development (27°C to 17°C) and somewhere after 60% for (17°C to 27°C), again representing instar III/ instar IV. By contrast in the switch-up protocol (27°C to 33°C or 33°C to 27°C) the TSP for sperm length was around instar I or 10% to 20% of development (33°C to 27°C) and between instar I and instar II or 20% – 40% of development (27°C to 33°C).

Why the TSP differs for switch-up and switch-down protocols is open to conjecture. When switching from 33°C to 27°C the TSP coincides with early embryonic development (the transition from zygote to the first larval instar). This period in ontogeny approximates the cellularisation of the pole plasm which goes on to form the primordial germ cells (Chapman, 1998). Thus, environmental perturbation at this stage may well disrupt this process and consequently affect sperm size. By contrast, switches from 27°C to 17°C revealed a TSP for sperm size expression around instar III, and instar IV. These instars are associated with gonad morphogenesis in a number of species (Belote & Baker, 1982; Brakefield *et al.*, 1996; Edgar, 2006). For example, in

the horn fly (*Haematobia irritans*), testes morphogenesis and the onset of spermatogenesis (up to metaphase I of the Ist meiotic division) takes place during the final larval instar (Basso *et al.*, 2011). Histological studies have also found spermatogenesis to occur during the final instar through to pupation in the South American fruit fly, *Anastrepha fraterculus* (Franceskin, 2005); the house fly, *Musca domestica* (Perje, 1948) and *D. melanogaster* (Demerec, 1994). Thus, it is perhaps not surprising a thermal switch at this point in development affects sperm size. Of interest, the two TSPs appear to be associated with critical periods of gonadal development and thus it is possible that cellularisation of the pole plasm is sensitive to elevated temperature perturbation whilst gonad morphogenesis is sensitive to reduced temperature perturbations. Indeed, genes involved in sexual differentiation (male and female gonad morphogenesis) such as the *transformer-2* locus are known to be heat sensitive. Thus, chromosomally females that experience a temperature switch from 16°C to 29°C develop male sexual organs because the *tra-2* locus is disabled (loses function) at elevated temperature (Belote & Baker, 1982).

Although the exact mechanisms that bring about the phenotypic plastic expression of sperm length and elytra length are unknown, it is likely that temperature acts as an external cue which alters the timing and duration of hormone synthesis during larval development (see, Nijhout, 2003b). In insects larval serum proteins (LSPs) are secreted into the haemolymph during the final instar where they are used in morphogenesis during pupation as an amino acid rservoir. LSP gene expression is regulated by 20-hydroxyecdysone (20-E), a hormone important in the regulation of larval development. In the Glanville Fritillary (*Melitaea cinxia*) LSP expression increased with increasing

temperature (which co-varied with development time). Thus it is possible temperature affects the expression of LSP either directly or indirectly (i.e. via an upstream regulatory pathway) which consequentially affects morphogenesis (Kvist *et al.*, 2013) and subsequently affects the organism's ability to produce gametes of the correct size. Indeed, in reptiles ESD appears to be mediated via the effect of ambient temperature on the synthesis of oestrogens, antioestrogens and aromatase inhibitors (that play a major role in converting androgen into oestrogen), which in turn affect gonadal development (Desvages *et al.*, 1993; Pieau, 1996; Pieau *et al.*, 1996; Pieau *et al.*, 2001; Pieau & Dorizzi, 2004).

In *D. melanogaster,* a thermal switch from 20°C to 29°C early during instar III resulted in decreased circulating levels of ecdysone, which caused these individuals to remain as a free-living larvae (Garen *et al.*, 1977). The same thermal switch at instar I resulted in pupation and eclosion, but the shift resulted in developmental defects causing sterility in females (Garen *et al.*, 1977). These females successfully mated at 29°C but due to decreased levels of ecdysone in the haemolymph, they laid defective eggs and eventually stopped laying eggs completely (Garen *et al.*, 1977). This may be connected to the heat-sensitive *transformer-2* gene; at 29°C *tra-2* gene experiences a loss of function, thus females switched to 29°C are less able to repress the development of male primordia (Belote & Baker, 1982).

To conclude, the temperature experienced during ontogeny affects morphological traits associated with reproduction in a number of species. Thermal-switch experiments allow us to identify at which point during development, organisms are particularly sensitive to temperature. Here, I have

shown there may be two TSP that affect the expression of sperm length in *C. maculatus*; one early in development and one during larval instar III/IV. These two periods appear to correspond to key ontogenic events. That these periods were identified separately from switch-down and switch-up experiments suggests the developmental pathways associated with the two events (pole plasm cellularisation and gonad morphogenesis) may be controlled by different genes that are differentially affected by environmental temperature. Tighter control over switch periods (i.e. switch-up or switch-down at closer intervals, perhaps every 10% into development) coupled with a histological study of gonad morphogenesis would allow a more complete and accurate picture of when during development sperm length expression is temperature sensitive. In the light of the observed effects of developmental temperature on sperm characteristics, I next examine the effect of thermal rearing temperature on subsequent reproductive behaviour.

5. The effect of thermal environment on copula duration.

5.1 Introduction

Copulation can be defined as engaging in sexual intercourse by the insertion of a male's reproductive organ into the female reproductive tract that facilitates the deposition of spermatozoa into the female's reproductive tract (Simmons, 2001; Oxford Dictionary of Science). The primary function of copula is the transfer of sperm and seminal fluid, although copulatory behaviour may serve other functions (Eberhard, 1996). In some species of insect copula duration can be difficult to measure because the distinction between copulatory phases that lack genital contact and copulatory phases that involve genital contact are hard to distinguish. Despite such difficulties, copulation duration is highly variable both within and between species with some of this variation representing functional adaptations, and some of this variation resulting from environmental influences such as temperature or mating history (Simmons, 2001).

The observation that females frequently mate with multiple males (Eberhard, 1996; Birkhead & Møller, 1998; Simmons, 2001) and the fact that females of many species store sperm, opens up the opportunity for females to bias paternity (Eberhard, 1996). These two characteristics (polyandry and female sperm storage) exert selection on males to protect/ensure paternity via a plethora of mechanisms (Parker, 1970a; Simmons, 2001). The adaptive significance of extended copulation has been extensively studied (reviewed by Simmons, 2001) and in general copula duration increases with increased sperm competition risk (Simmons, 2001). From a male perspective, this could

be to i) increase the level of sperm preemption or ii) reduce the likelihood of female remating (mate guarding). For example, in the yellow dung fly (*S. stercoraria*), male fertilization success increases with increasing copulation duration as a result of increased levels of sperm displacement (Parker *et al.*, 1999). In the soapberry bug (*Jadera haematoloma*), male-biased adult sex ratios dictate that males face fierce competition over mating from rivals and thus successful males guard their mates by remaining in copula, which can last for up to 11 days in this species (Carroll, 1991).

Males may also use copulation to elicit favourable physiological responses from females, *sensu* cryptic female choice (Eberhard, 1996). Thus males could increase their reproductive success if copulatory behaviour delays female receptivity to further matings with rival male, stimulate the uptake, storage and use of self's sperm, stimulate the ejection of previously stored, rival male sperm, and/or accelerate vitellogenesis and oviposition (Eberhard, 2011). For example, in the golden egg bug (*Phyllomorpha laciniata*), elevated sperm competition risk resulted in an increase in copula duration and rate of sperm transfer (García-González & Gomendio, 2004). Here, the increase in copula duration appears to function to increase the likelihood of female oviposition soon after copulation, in order to maximize the male's chances of siring at least some eggs before the female remates and the male's sperm are preempted.

The effect of temperature on copulation duration is well known and in light of the observation that copula duration was considerably longer at lower temperatures (Parker 1971), Parker (1970a, b, c) was careful to correct the copulation duration of dungflies to 20°C in his pioneering work on sperm

competition and optimality theory. More recently a number of studies have examined the effect of temperature on copulatory behaviour (McDonald & Nijhout, 2000; Switzer *et al.*, 2008; Jiao *et al.*, 2009; Katsuki & Miyatake, 2009; Chen *et al.*, 2010). For example, in the wolf spider (*Pardosa astrigera*), latency to copulate and the duration of copula, both increased with decreasing temperature, whilst the frequency of pedipalp insertions and frequency of hematodochal expansions decreased with decreasing temperature (Jiao *et al.*, 2009). In addition, females that copulated at elevated temperatures produced more egg sacs, possibly due to these females receiving greater quantities of seminal fluid and thus more ovulation promoters during copulation (Chen *et al.*, 2010). In the Japanese beetle (*Popillia japonica* Newman), mating duration and mate guarding is a function of ambient temperature. Copulations taking place at high (32°C) temperatures resulted in shorter copulation durations in comparison to lower ambient temperature (Switzer *et al.*, 2008). This pattern was even evident when males copulated with dead females, suggesting males are the main arbiter of copula duration in this species. Interestingly, in this species food reserves provide both water and energy for thermoregulation and paired males were found to have lower water content than single males due to reduced feeding opportunities (Saeki *et al.*, 2005). In the bruchid beetle, *C. chinensis,* a sister species of *C. maculatus,* Katsuki & Miyatake (2009) found a negative relationship between ambient temperature and copula duration. They also found that these longer copulations were associated with greater sperm transfer. However, they estimated sperm transfer as the number of sperm in the spermatheca, which is the site of sperm storage and does not necessarily equate to the number of sperm transferred (Eady:

personal communication). Thus this latter finding needs to be verified. Female remating frequency was also affected by the ambient temperature at the time of the first mating; remating frequency was lower when initially mated at 17°C in comparison to those initially mated at 25°C or 33°C. This was attributed to greater sperm transfer at 17°C. In some Calopterygid damselflies, females preferred males with elevated thorax temperatures. Longer periods of solar exposure increased male thorax temperature which influenced the frequency of wing-beat which in turn functioned as a cue for the female to detect and choose a mate (Tsubaki *et al.*, 2010).

Despite a growing number of studies reporting an effect of ambient temperature on copulation duration, to my knowledge few have examined the effects of larval rearing temperature on subsequent copulatory behaviour. Given larval developmental temperature affects spermatogenesis, I here predict that larval rearing temperature will affect copula duration. Specifically, I predict that when larvae develop at both high and low temperatures, copulation duration will increase because at these temperatures males transfer fewer sperm and there is good evidence in *C. maculatus* that sperm transfer and copulation duration are negatively related: sperm limited males copulate for longer (Brown, 2001).

5.2 Materials and Methods

5.2.1 Effect of post-eclosion temperature on copula duration

The effect of post-eclosion temperature on copula duration was investigated by rearing (from eggs) larvae in the insectary at 27°C, 32% RH. Approximately 1000 adults (estimated by mass) were housed with 200g of

moth beans (~ 7500 beans) for one hour at 27°C. The egg-laden beans were then separated into four triple vent 110 mm Petri dishes, with approximately 1500 beans per Petri dish. The Petri dishes were randomly positioned within the insectary environment to minimize the likelihood of micro-climate effects. Just prior to adult eclosion, the beans were transferred into individual cells of a 25 cell replidish and sealed with a glass lid, from which emergent beetles could be collected and adult virginity ensured. Adults (0 – 2h old) were randomly transferred into separate incubators (Lucky Reptile Herp Nursery II, The reptile shop, UK), and maintained at 17°C, 25°C, 27°C and 33°C for 24 – 48h. Males and females were paired at either at 17°C, 25°C, 27°C or 33°C and the duration of copulatory phases recorded. Copulation in *C. maculatus* can be differentiated into two phases: the start-to-kick phase, this is when ejaculates are transferred to the female, and the kick-to-end phase, where the female kicks at the male with her hind legs in an attempt to dislodge the male, before terminating copulation (Eady, 1994a; Edvardsson & Canal, 2006).

5.2.2 Effect of larval rearing temperature on copula duration

The effect of larval rearing environment on copulation duration was investigated by rearing (from eggs) larvae at 17°C, 25°C, 27°C and 33°C. Prior to eclosion, egg-laden seeds were plated out into replidishes to ensure the virginity of emergent adults. Post emergence, the adults were sexed and maintained at the same environmental temperature as the larvae until 24 – 48h old. Virgin males and females from the same environments were then paired at 27°C (within the insectary), and copulation duration recorded as above. The parametric data presented here was analyzed for both deviations from normal distributions and for heterogeneous variances between the various treatment

groups. All behaviours were analysed using one-way ANOVA on SPSS version 20 (IBM) and significant ($P<0.05$) differences between groups were established using *Post-hoc* tukey test.

5.3 Results

The post-eclosion temperature at which copulation took place significantly affected copula duration. The start-to-kick phase was longest at 17°C with the duration of this phase being equivalent at 25°C, 27°C and 33°C (ANOVA $F_{3,58} = 23.547$, $P < 0.0001$, Fig. 20). The kick-to-end phase was unaffected by post-eclosion temperature (ANOVA $F_{3,58} = 1.179$, $P = 0.326$, Fig. 21) although, total copulation duration was longest at the lowest post-eclosion temperature (ANOVA $F_{3,58} = 15.176$, $P<0.0001$, Fig. 22).

The thermal environment at which egg-to-adult development took place affected copulatory behaviour. The start to kick phase was longest for those beetles reared at 17°C in comparison to those reared at 25°C, 27°C or 33°C (ANOVA $F_{3,80} = 7.897$, $P < 0.0001$, Fig. 23). The kick-to-end phase was unaffected by larval rearing environment (ANOVA $F_{3,80} = 0.145$, $P = 0.933$, Fig. 24) but in combination the total copulation duration was greatest when larvae were reared at the lowest temperature (ANOVA $F_{3,80} = 5.829$, $P < 0.001$, Fig. 25).

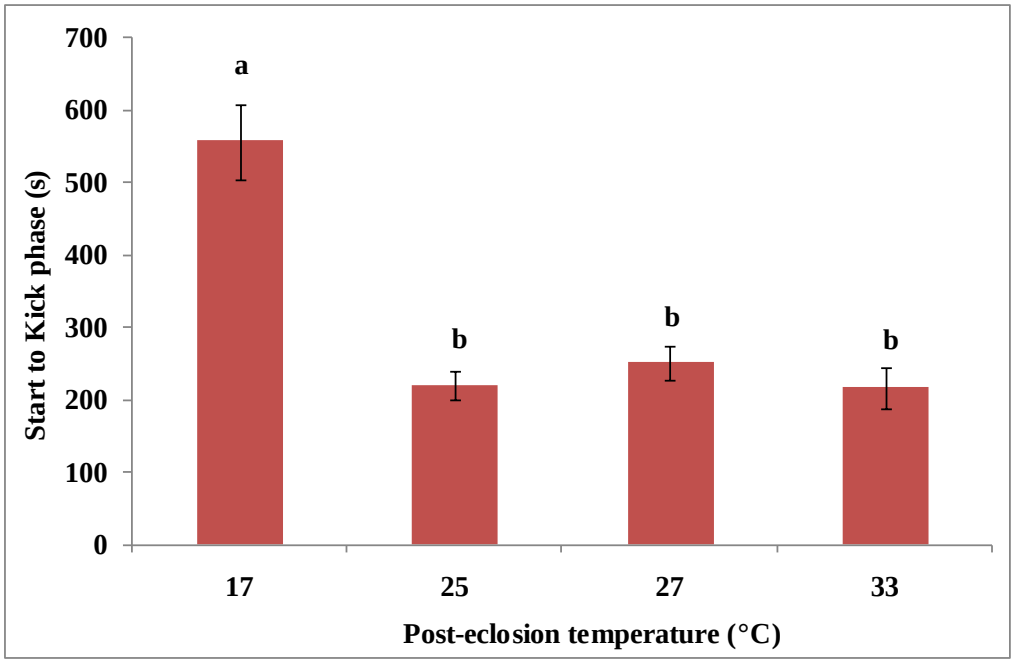


Fig. 20 Mean (±) standard error duration of the start-to-kick phase measured at 4 different post-eclosion temperatures. Bars with different superscripts indicate significantly different temperature effects as revealed by *Post-hoc* tukey test.

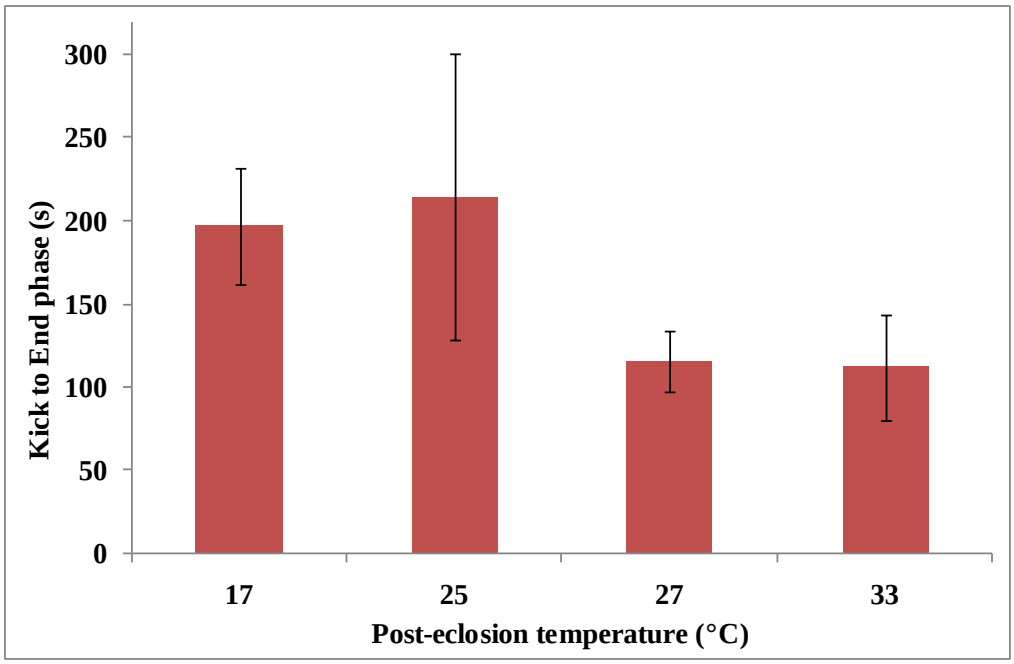


Fig. 21 Mean (±) standard error duration of the kick-to-end phase measured at 4 different post-eclosion temperatures.

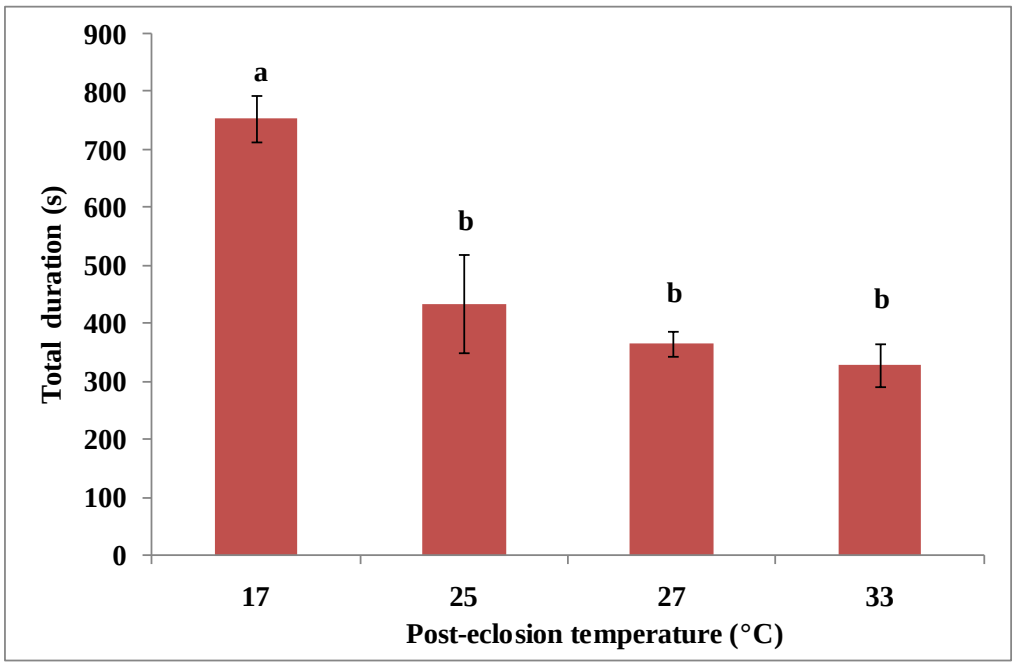


Fig. 22 Mean (±) standard error total copulation duration in relation to 4 different post-eclosion temperatures. Bars with different superscripts indicate significantly different treatment effects as revealed by *Post-hoc* tukey test.

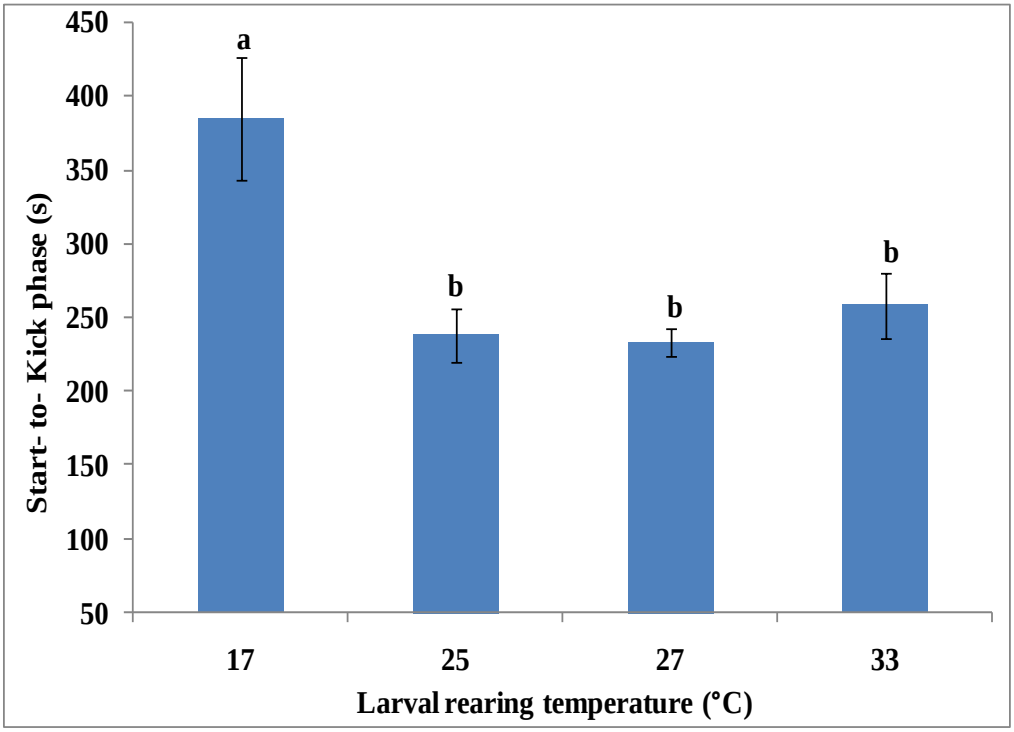


Fig. 23 Mean (±) standard error of the start-to-kick phase in relation to 4 different larval rearing temperatures. Bars with different superscripts indicate significantly different groups as revealed by *Post-hoc* tukey test.

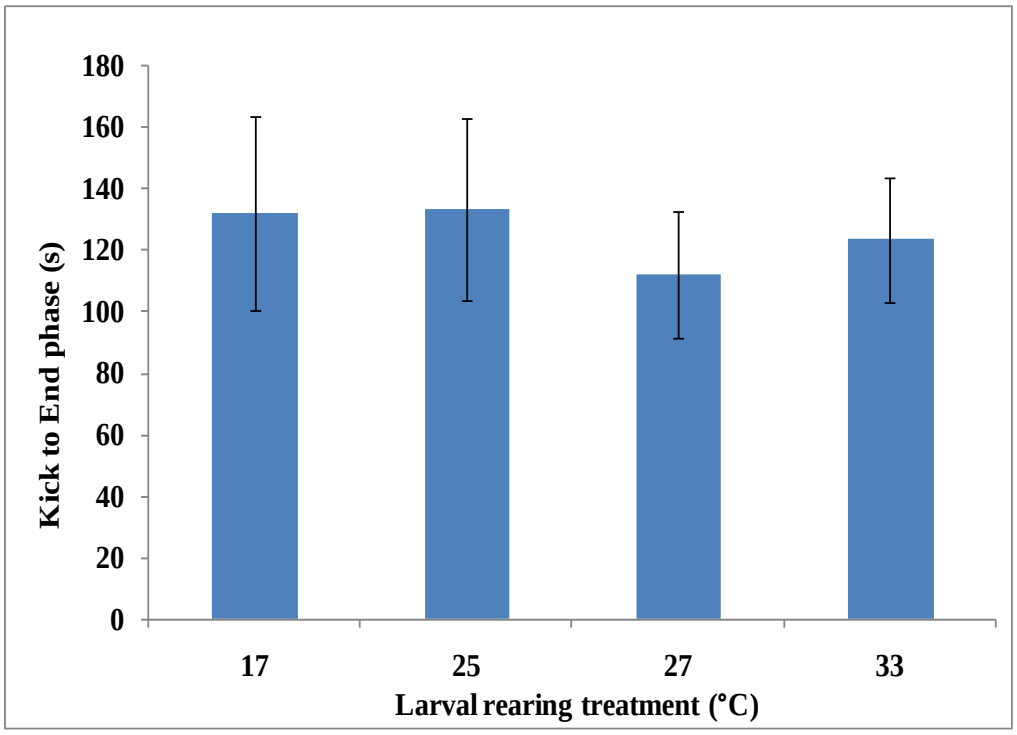


Fig. 24 Mean (±) standard error duration of the kick-to-end phase of copulation at 4 different larval rearing temperatures.

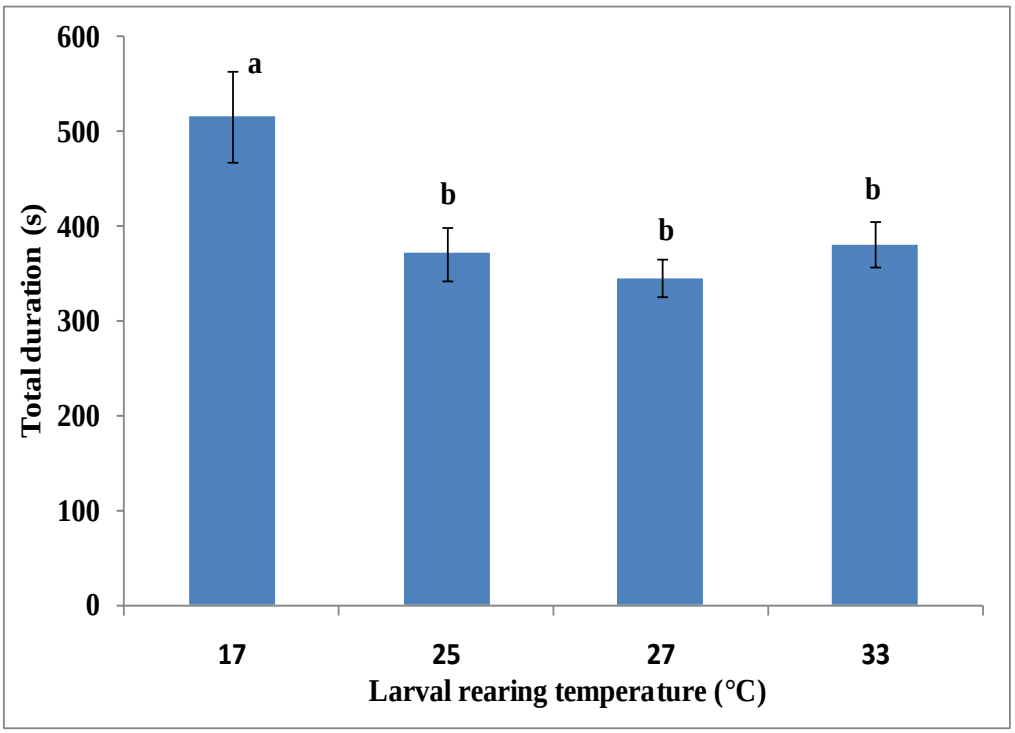


Fig. 25 Mean (±) standard error total copulation duration when larvae were reared at 4 different temperatures. Bars with different superscripts indicate significantly different treatment effects as revealed by *Post-hoc* tukey test.

5.4 Discussion

Although previous studies have shown an effect of larval rearing substrate on latency to copulation (Brazner & Etges, 1993) whilst others have shown an effect of developmental temperature on male nuptial gift size (Fox *et al.*, 2006) and female fecundity and egg size (Stillwell & Fox, 2005), to my knowledge this is the first study to demonstrate an effect of larval rearing temperature on subsequent adult copulatory behaviour. Copulation duration was longest for those larvae reared at the coolest temperature (17°C). This increase in the duration of copulation is almost entirely due to an increase in the phase I of copulation. Interestingly, larval rearing and post-eclosion temperature had no effect on the kick-to-end phase of copulation.

In *C. maculatus*, sequentially mating males to females results in a dramatic decline in ejaculate size (Fox *et al.*, 1995; Savalli & Fox, 1999) and the number of sperm transferred (Eady, 1995). Brown (2001) subsequently demonstrated that the sequential mating of males resulted in a significant increase in copulation duration of *C. maculatus*, that was almost entirely due to an increase in the first phase of copulation. Thus males that are sperm (or ejaculate) limited take longer to elicit the kicking behaviour of females (Eady, 1995), possibly due to the decreased activity of stretch receptors in the wall of the bursa copulatrix (where the ejaculate is received; Eady, 1994) which possibly function to signal when to terminate copulation. Thus, sperm limited males take longer to fill the bursa copulatrix and thus longer to elicit the kicking behaviour of the female. That males reared at 17°C transferred significantly fewer sperm at copulation than males reared at other temperatures (chapter 3), supports the notion that elicitation of kicking

behaviour (Phase II) is related to sperm/ejaculate transfer. Brown (2001) found no effect of sequential mating and hence sperm limitation on phase II of copulation (kick-to-end), supporting the notion that the duration of second phase is unrelated to sperm transfer. The non-significant effect of larval rearing temperature on the second phase of copulation reported here is in agreement with this proposition.

In the present study copulation duration increased at 17°C. This appears to be entirely due to an increase in the first phase of copulation and it is surprising that post-eclosion temperature did not affect the second phase (kick-to-end) of copulation, given that metabolic rate and thus the activity of poikilotherms is temperature sensitive (Nijhout, 2003b; Kingsolver *et al.*, 2004b; Angilletta, 2009). The inverse relation between temperature and phase I of copulation may reflect the fact that this is when ejaculate transfer takes place. Thus, the rate at which sperm and seminal fluid are transferred is likely to decrease with decreasing temperature and, in line with earlier arguments, is likely to result in a longer latency to female kicking. The transfer of seminal fluid at low temperatures could be constrained by physiology (metabolism slows at lower temperature) and/or fluid viscosity; fluids become more viscous at lower temperatures (Purchase *et al.*, 2010). Why post-eclosion temperature had no effect on the duration of phase II of copulation is surprising given the effect of temperature on metabolism and behaviour. One possible explanation as to why the 2nd phase of copula was unaffected could be that the termination of copulation is under the influence of both male and female attributes. Thus, at low temperatures females kick with less intensity/frequency but the male's

ability to maintain genital contact is also reduced, producing an overall neutral effect of temperature on the duration of phase II of copulation.

An increase in phase I and no effect on phase II of copulation, results in an extended total copula duration at lower temperatures. A number of studies have shown similar results; Horton *et al.* (2002) found copulation duration to increase with decreasing temperature in three species of predatory bug belonging to the Anthocoris genus. In the wolf spider (*Pardosa astrigera*), courtship latency, courtship duration and mating duration all increased with decreasing temperature (Gering, 1953; Rovner, 1971).

Katsuki & Miyatake (2009) demonstrated a negative association between ambient temperature and copulation duration in *C. chinensis.* Of interest, the longer copulations at the lower temperatures were associated with increased sperm transfer and reduced female receptivity to further copulation. Katsuki & Miyatake (2009) argue that increased number of sperm transferred at 17°C (measured as the number of sperm in the spermatheca) delays female receptivity to further copulation, a mechanism similar to that proposed by Savalli & Fox (1998) and Eady (1995) in the bruchids *Stator limbatus* and *C. maculatus* respectively. However, Eady (personal communication) believes the authors have not measured the number of sperm inseminated because the ejaculate is transferred to the bursa and not the spermatheca in *C. chinensis*. Thus interpretation of Katsuki and Miyatake (2009) is difficult.

In numerous arthropod species longer copulations allow males to transfer ejaculatory substances other than gametes in order to manipulate female reproductive output and behaviour (Arnqvist & Nilsson, 2000). For example, in redback spiders (*Latrodectus hasseltii*), males can be cannibalized

by the female during copulation, which results in a paternity advantage over males that are non-cannibalized (Snow & Andrade, 2004). Cannibalized males mated for longer than non-cannibalized males (19 minutes vs. 13 minutes) and managed to transfer more sperm (~ 54,000 sperm as opposed to ~ 33,000 sperm). However, sperm transfer in this species is more or less complete after ~ 5 minutes of copulation, thus the function of longer copulation is not clear. It could be that cannibalistic females somehow determine how many sperm get transferred or longer copulations may be an adaptation to transfer non-gametic substances to the female that either delay female receptivity, stimulate oviposition or somehow assist in sperm displacement (Wolfner, 1997). Alternatively, it has been suggested that longer copulations may also serve as a stimulus wherein females cryptically select males with extended copulations leading to a potential bias in sperm storage/use, although this is hard to demonstrate (Eberhard, 1996; Snow & Andrade, 2004). In some species, copulation duration is associated with greater paternity in accordance with the ejaculate transfer hypothesis (e.g. Simmons, 2001 pg.198). However, this is unlikely to be the case in the present study because sperm limited males copulated for longer (see also Brown, 2001). Thus, in general and especially in nature where developmental temperature is likely to be variable, (see below), the relationship between copulation duration and sperm transfer is unlikely to be straightforward. This is further complicated by the fact that "it takes two to tango". Essentially copulation duration is likely to be in part male derived, in part female derived and in part the interaction between male and female traits (Brown, 2001). To test this further it would be useful to record the copulation duration of males reared at different temperatures when mated to a standard

female (i.e. females reared at 27°C- insectary conditions) and vice-versa (i.e. females reared at different temperatures and mated to a standard insectary male). This would shed light on which sex exerts the greatest control over copulation duration.

The effect of developmental temperature on reproductive parameters, including copulation duration is relevant because in nature there are considerable temperature fluctuations. For example, in Finland, dung pat temperatures can range from 10°C – 35°C in a matter of days (Penttilä *et al.*, 2013). Thus, dung fly larvae will be exposed to a wide range of temperatures during their development which could impact on their subsequent reproductive anatomy and physiology (Blanckenhorn, 2000; Blanckenhorn & Hellriegel, 2002; Blanckenhorn & Henseler, 2005). This is likely to contribute to variation in copulation duration and could potentially explain some of the additional unexplained variation around predicted copulation duration optima (Parker & Simmons, 1994). Thus the role of developmental temperature on subsequent adult copulatory behaviour should be investigated in a range of species.

6. Effect of larval rearing temperature on sperm performance during postcopulatory sexual selection

6.1 Introduction

Parker's revolutionary idea was that sexual selection continued beyond the male's precopulatory struggle for access to females and into the postcopulatory arena (Parker, 1970a,b,c). Parker termed this postcopulatory competition for fertilization, "sperm competition": the competition between the sperm of two or more males for the fertilization of the female's ova (Parker, 1970a,b,c). Since then, sperm competition has been recognised as a pervasive selection pressure that has resulted in the evolution of a wide range of physiological, morphological and behavioural traits that serve to enhance a male's fertilization success in the face of competition from other males (Parker, 1970a,b; Birkhead & Møller, 1998; Simmons, 2001). In insects the risk of sperm competition is one where females mate more than once during a single reproductive cycle and can store sperm in specialized sperm storage organs known as spermathecae (Parker, 1970a,b,c; Birkhead & Møller, 1998; Ridley, 1988; Simmons, 2001).

Females are known to use the sperm of more than one male following multiple inseminations, with the outcome generally expressed as a P_2 value; the proportion of offspring sired by the second male to mate (Boorman & Parker, 1976), which, in experimental studies, is usually the last male to mate. Values of P_2 reflect patterns of sperm use and can provide an insight into the underlying mechanisms of sperm competition. For example, a high P_2 value might indicate sperm stratification within the female's sperm stores, such that

the last sperm to enter the stores are the first to exit, giving rise to second male sperm precedence. Alternatively it could indicate the removal of rival sperm from the female reproductive tract, either by flushing previously stored sperm from the female sperm storage organ or by the mechanical removal of previously stored sperm (Waage, 1979; Ono *et al.*, 1989; Eady, 1994a,b; Birkhead & Møller, 1998; Simmons, 2001). Intermediate values of P_2 tend to indicate sperm mixing (Birkhead & Møller, 1998), whilst low P_2 values suggest first male sperm precedence. First male sperm precedence that could arise through a "topping off" mechanism in which the female's spermathecae is partially (mostly) filled with sperm from the first male to mate, such that a subsequent male can only add relatively few sperm to the spermatheca. The 'topping off' is complete when the sperm from these two (or more) males mix within the spermathecae prior to fertilization (Jones *et al.*, 2002). Under such conditions the first male to mate is likely to have an advantage in sperm competition. A possible example is in the rough skinned newt (*Taricha granulosa*); males deposit spermatophores on the substrate which is subsequently picked up by the female. Microsatellite analysis revealed the first male to mate sired the majority of the offspring (Jones *et al.*, 2002).

Sperm competition in *C. maculatus,* was first studied by Eady (1991) using both the sterile male and genetic marker technique. With a 24h interval between matings, $P_2 = 0.83$. A similar level of last-male sperm precedence ($P_2 = 0.9$) has been recorded in the bruchid beetle, *Bruchidius dorsalis* (Takakura, 2001), whilst in *C. chinensis,* first-male sperm precedence ($P_2 = 0.25$) has been observed (Harano *et al.*, 2008). In *C. maculatus*, variation in P_2 was unrelated to variation in male size, female size nor copulatory behaviour and males were

shown to be unable to directly remove previously inseminated sperm (Eady, 1994a). However, P_2 was shown to drop from approximately 0.85 to 0.55 when female oviposition was restricted between matings, prior to the second copulation (Eady *et al.*, 2004), suggesting P_2 to be, in part, determined by sperm depletion from the spermatheca. In *C. maculatus,* virgin males transfer 50,000 – 60,000 sperm at copulation, which is approximately 85% more sperm than females can store in their spermathecae (Eady, 1994a). Although theoretical models of sperm displacement (Parker *et al.*, 1990; Parker & Simmons, 1991) predict last male sperm precedence in this species (Eady, 1995), a lack of detailed knowledge about how sperm enter and exit the female's spermatheca means that the exact mechanism of sperm precedence in this species remains unknown.

Wilson *et al.* (1997) provide evidence that the female genotype influences sperm precedence in *C. maculatus*; P_2 values of male pairs were highly repeatable when mated to separate full-sib females but were either non-repeatable or marginally repeatable when male pairs were mated to two unrelated females. This suggests male success at sperm competition is strongly influenced by male-female compatibility (also shown in *D. melanogaster*; Clark *et al.*, 1999). However, the underlying mechanisms of male-female compatibility remain unknown.

Whether variation in sperm size in *C. maculatus* explains some of the variation in P_2 has yet to be tested. In the field cricket (*Gryllus bimaculatus*), sperm length is thought to be a sex-linked trait and lines were artificially selected to produce males with short, medium and long sperm length (Morrow & Gage, 2001a; 2008). When sperm of different lengths were competed

against each other, Morrow & Gage (2001b), initially found no evidence that sperm size affected P_2 in this species. Subsequently, Gage & Morrow (2003) examined the effect of sperm length on P_2 after correcting for covariance in sperm number and found that shorter sperm were more successful during competitive matings than longer sperm. A small-sperm advantage was also reported in the malaria vector (*Anopheles gambiae*); males producing shorter sperm sired a higher proportion of offspring than males producing longer sperm. Insemination success, sperm motility and the proportion of ovipositing females decreased with increasing sperm length in this species (Voordouw *et al.*, 2008). In the dung beetle (*Onthophagus taurus*) shorter sperm outcompeted longer sperm during sperm competition, although this was partly dependent on female reproductive tract morphology (García-González & Simmons, 2007). In sum, these results are in accordance with theoretical models of male gametic evolution under conditions of sperm competition, in which males are predicted to produce numerous, tiny sperm (Parker, 1982).

However, there are numerous cases in which males appear to produce fewer, larger sperm in the face of sperm competition. In the bulb mite (*Rhizoglyphus robini*), males produce aflagellate, ameboid-type sperm that move with the help of pseudopodia. Males producing larger sperm were more successful in sperm competition, with 15% of the variation in fertilization success explained by variation in sperm size (Radwan, 1996). The reason for the competitive advantage of larger sperm at fertilization is not fully understood, although large ameboid cells could be at an advantage in resisting osmotic changes or have greater longevity in the female's seminal receptacle. A similar result was found in the hermaphroditic nematode (*Caenorhabditis*

elegans); larger sperm (again ameboid) outperformed smaller sperm during sperm competition, possibly through crawling faster and displacing smaller, hermaphrodite sperm in the process. However, longer sperm were found to be costlier to produce and take longer to develop than small sperm, perhaps constraining their production (LaMunyon & Ward, 1998). In the fresh water snail (*Viviparus ater*), distinct nonfertile, oligopyrene sperm are produced. Their function is still not clear but they are believed to complement the eusperm (fertile sperm) at fertilization. In this species, males that produced longer oligopyrene had a greater success during sperm competition, with the size of the oligopyrene sperm being positively correlated with male fertilization success (Oppliger *et al.*, 2003).

The female reproductive environment is also likely to be a potent selection pressure on sperm morphological evolution (Wilson *et al.*, 1997). Thus, where larger sperm function better in the female reproductive tract, then the production of tiny sperm (Parker, 1982) may be selected against. There is ample comparative evidence that sperm morphology coevolves with dimensions of the female reproductive tract (Dybas & Dybas, 1981; Presgraves *et al.*, 1999; Morrow & Gage, 2000; Minder *et al.*, 2005; Rugman-Jones & Eady, 2008). Within species, Miller & Pitnick (2002) found that longer sperm males outperformed short-sperm males in sperm competition trials only when the competition took place in the reproductive environment of females with long seminal receptacle lengths. Long-sperm males did not perform better or worse than short-sperm males within short seminal receptacle length females. In addition, in lines selected for long seminal receptacle length in females, there was a correlated evolution of sperm size;

males from these lines had longer sperm. This correlated evolution is consistent with good genes and runaway (Fisherian) sexual selection (Andersson, 1994).

Thus the outcome of sperm competition has been shown to depend on a complex of interacting terms, including sperm number, sperm size and the interaction between these male traits and the female reproductive tract. Given we know that sperm number and sperm size to be influenced by developmental temperature, I here predict that the developmental temperature experienced by males will influence the outcome of sperm competition. The outcome of sperm competition was estimated using the genetic marker technique using tan and black beetles (Black females: B♀ & Black males: B♂) (Eady, 1991). Specifically I predict males that undergo development at the temperature extremes will perform poorly in terms of sperm competition in comparison to males reared at intermediate temperature (27°C). This is because the population has been maintained at ~ 27°C for ~ 15 years, which represents ~ 200 generations of selection for performance to be optimised at this temperature. The effect of developmental temperature on the outcome of sperm competition is also relevant, because in nature, the thermal environment experienced by developing organisms (especially ectotherms) varies widely. For example, Kvist *et al.* (2013) report Glanville Fritillary (*M. cinxia*) larvae to experience temperature fluctuation from 10°C to 30°C. However, only a few sperm competition studies have considered variation in ontogenic environment on the outcome of sperm competition (Amitin & Pitnick, 2007) and none (to my knowledge) have considered variation in ontogenic

temperature. Here I present the first study to report an effect of developmental temperature on the outcome of sperm competition.

6.2 Materials and Methods

Approximately 1000 wild-type adults (estimated by mass) were housed with 200g of moth beans (*Phaseolus aconitifolius*) (~ 7500 beans) for one hour at 27°C, 32% RH in the insectary. The egg-laden beans were then separated into triple vent 110mm Petri dishes (Fisherbrand, www.fisher.co.uk) with approximately 2000 beans per Petri dish. These were housed in six separate incubators (Lucky Reptile Herp Nursery II, The reptile shop, UK), two at 17°C, two at 27°C & two at 33°C. Just prior to adult emergence, the beans containing developing larvae were plated out into individual cells of a 25 cell replidish and sealed with a glass lid. Petri dishes were checked for emergence every 24h to obtain virgin adults of known age (24 – 48h post eclosion). These treatment males ($T_{17°C}$, $T_{27°C}$ & $T_{33°C}$) were used in sperm competition experiments (Eady 1991).

Black-line females were mated to experimental (wild-type) males and black-line males. Black-line beetles (virgins of known age) were obtained as above, except all black-line beetles completed their development at 27°C in the insectary. Male and female black-line morphs of *C. maculatus* are completely black apart from white pubescence on the elytra, pronotum and pygidium. Crosses involving a black female x black male, result in black offspring. Black female x wild-type male, results in dark-tan offspring, with distinctive tan forelegs and tan antennae (excepting the first 2 – 4 antennal segments) (Eady, 1991).

All beetles used in the experiment described below were between 24 – 48h from eclosion and all matings took place in the insectary at 27°C. The experiment ran over 8 weeks because of the large difference in development time of beetles reared at 17°C, 27°C & 33°C. In order to have an uninterrupted supply of virgin black beetles, batches of fresh moth beans were housed with the black-line stock culture every four days, in order to stagger beetle eclosion over an eight week period.

6.2.1 Experimental Design

6.2.1.1 Effect of developmental temperature on sperm offence (P_2)

Virgin black females were permitted a single copulation with a virgin black male. Following copulation the male was discarded and females housed in individual Petri dishes containing 30 moth beans for 24h prior to being remated to a wild-type male from one of the experimental treatments: $B_{♀}$ x $B_{♂}$ x $T_{♂}$ in which $T_{♂}$ was derived from either $T_{17°C}$, $T_{27°C}$ or $T_{33°C}$. After the second copulation the wild-type male was discarded and the female transferred to a Petri dish containing 30 fresh moth beans on which to oviposit until her natural death. Twenty replicates each of $T_{17°C}$, $T_{27°C}$ and $T_{33°C}$ were obtained. The egg-laden beans were incubated at 27°C in the insectary until the offspring emerged.

6.2.1.2 Effect of developmental temperature on sperm defence (P_1)

Virgin black females were permitted a single copulation with a wild-type male from either $T_{17°C}$, $T_{27°C}$ or $T_{33°C}$. Following copulation the male was discarded and females housed in individual Petri dishes containing 30 moth beans for 24h prior to being remated to a wild-type male. Females were remated to a virgin black male ($B_{♀}$ x $T_{♂}$ x $B_{♂}$). Following the second

copulation the black male was discarded and the female allowed to oviposit on 30 fresh moth beans until her natural death. Eighteen replicates each of the $T_{17°C}$, $T_{27°C}$ or $T_{33°C}$ were obtained. The egg-laden beans were incubated at 27°C in the insectary until the offspring emerged.

Two weeks after offspring emergence, the Petri dishes were sealed with electrical tape and moved to a freezer at -5°C for 48h in order to euthanize any live beetles. Mean P_2 and P_1 values were calculated from the ratio of black to non-black offspring.

6.2.2 Statistical analysis

Analyses of proportion data were carried out using a generalised linear model (GLM) with binomial errors and *logit link* function (Crawley, 2002), using the statistical package S-plus. A non-parametric test, Kruskal-Wallis test, was used to investigate offspring counts as the number of counts per sample (temperature treatment) was different among the different rearing temperatures.

6.3 Results

6.3.1 The effect of developmental temperature on sperm offence (P_2)

The mean (± SE) number of offspring produced following $B_{♀}$ x $B_{♂}$ prior to mating to $T_{17°C}$, $T_{27°C}$ & $T_{33°C}$ males were 27 ± 1, 27 ± 1 & 26 ± 2 (Kruskal-Wallis X^2 = 0.04, df = 2, P = 0.98). The developmental temperature experienced by the wild type males had a significant effect on P_2 (Δ deviance = 290, $F_{2,69}$ = 23.2, P < 0.0001). *Post-hoc* analyses (collapsing of factor levels) revealed males that underwent development at 17°C and 33°C had significantly reduced P_2 in comparison to males reared at 27°C (Fig. 26).

6.3.2 The effect of developmental temperature on sperm defence (P_1)

The mean (± SE) number of offspring produced following $B_{♀}$ x $T_{♂,}$ where the males came from $T_{17°C}$, $T_{27°C}$ or $T_{33°C}$ prior to mating to a $B_{♂}$ were 22 ± 3, 26 ± 3 & 26 ± 1 (Kruskal-Wallis X^2 = 4.4, df = 2, P = 0.11). The developmental temperature experienced by the wild type males had a significant effect on P_1 (Δ deviance = 109, $F_{2,62}$ = 7.3, P = 0.0014). *Post-hoc* analyses revealed males that underwent development at 17°C had a significantly reduced P_1 value in comparison to males reared at 27°C or 33°C (Fig. 27).

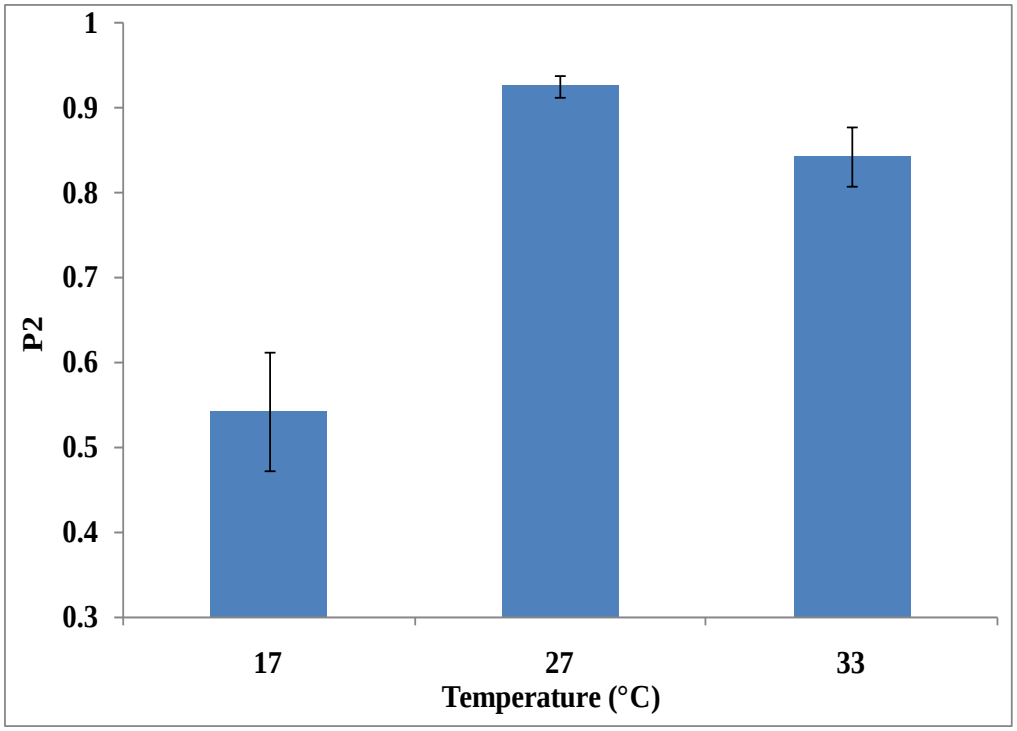


Fig. 26 Mean (± standard error) P_2 in relation to larval rearing temperature.

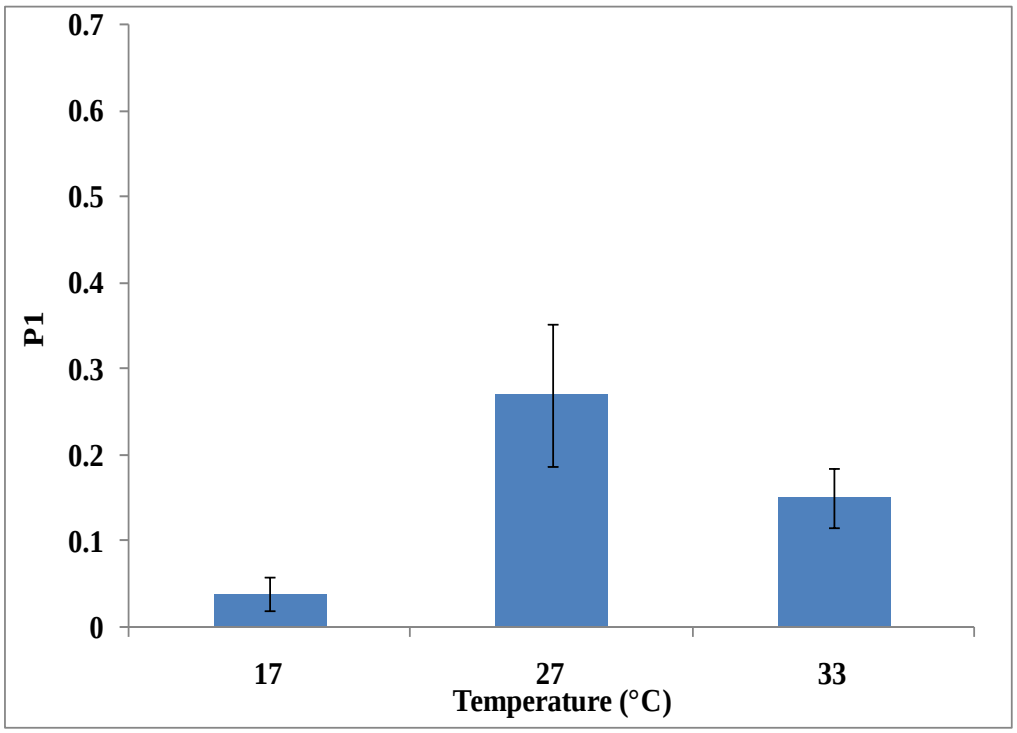


Fig. 27 Mean (± standard error) P_1 in relation to larval rearing temperature.

6.4 Discussion

To my knowledge, this is the first study to show the outcome of sperm competition to be influenced by thermal variation in the ontogenic environment. Males that experienced the two temperature extremes, 17°C or 33°C, during larval development were poorer at sperm offence than males reared at 27°C. Males that developed at 17°C were also poorer at sperm defense. Males reared at 27°C had the longest sperm (see chapter 3) in comparison to the males reared at 17°C & 33°C and so sperm length could explain some of the variation in the outcome of sperm competition. For example, nematode (*Caenorhabditis elegans*) LaMunyon & Ward (1998), bulb mite (*Rhizoglyphus robini*) Radwan (1996) and snail (*Viviparus ater*) Oppliger *et al.* (2003) all found longer sperm to have enhanced fertilization success during sperm competition. Longer sperm may be advantageous during sperm competition because they have greater longevity and swim at a greater velocity (Gage *et al.*, 2004; Snook, 2005; Rudolfsen *et al.*, 2008). However, *C. maculatus* males reared at 17°C had similar sized sperm to those reared at 33°C (chapter 3), yet the 17°C males had both a lower P_1 and P_2 than the 33°C males, suggesting that outcome of sperm competition is not based on sperm size alone. Indeed, males reared at 17°C copulated for longer and transferred fewer sperm than those reared at 27°C or 33°C. Thus, variation in copula duration, sperm transfer and/or sperm size could all contribute to the lower sperm competitive ability of males reared at 17°C.

In the *C. maculatus*, the second male to mate attains a P_2 of approximately 0.83 (Eady, 1994b), which is thought to be a consequence of a combination of passive sperm loss from the female's sperm stores between

matings and indirect sperm displacement (sperm flushing) (Eady, 1994a; Eady, 1995). In the current study where both the first and second mating males were reared at 27°C, P_2 = 0.89, which is close to that previously reported for this species. Males are known to transfer around 85% more sperm than females can store in their spermatheca, with many 'excess' sperm remaining in the bursa copulatrix where they appear to get digested (Eady, 1994b). Stockley *et al.* (1997), found that in fish with external fertilization, males that transferred more sperm had greater success in sperm competition. Similar results have been reported in mammals (Møller, 1988, 1989; also see review Birkhead & Møller, 1998) and crickets (Gage & Morrow, 2003). However, Eady (1995) found in *C. maculatus*, a reduction in the number of sperm inseminated by the first male to mate from approximately 56,000 to 8,700 (an 84% reduction in the number of sperm inseminated), had no effect on P_1, whilst a reduction in the number of sperm transferred by the second male to mate resulted in a slight but significant reduction in P_2 (from 0.95 to 0.78). Interestingly, the number of sperm inseminated at 17°C was an ~ 82% (replicate 1) or 42% (replicate 2) reduction in comparison to the number of sperm transferred by males reared at 27°C (chapter 3) yet, P_2 levels for the 17°C males were smaller than those reported by Eady (1995) for sperm limited males (8700 sperm resulted in a P_2 of 0.78). Thus, the effects of development on P_1 and P_2 are unlikely to be solely due to variation in the number of sperm transferred.

To date, longer copulation durations have been generally associated with increased fertilization success (Arnqvist & Danielsson, 1999a; Andrés & Rivera, 2000; Simmons 2001). Until now, no study has reported an inverse

relationship between copulation duration and fertilization success during sperm competition, although this appears to be the case here. In *C. maculatus*, Eady (1995) found the number of sperm transferred decreased with male mating history and Brown (2001) found copulation duration to increase with male copulation history suggesting that sperm limited males copulated for longer. Therefore, it is likely males reared at 17°C take longer to transfer fewer sperm at copula, and thus suffer lower P_1 and P_2, although the reduction in P_1 and P_2 is over and above that predicted on sperm numbers alone. In the absence of experimental manipulation of sperm number, Eady (1994a) found no association between copulation duration and P_2 in *C. maculatus*.

Ontogenetic temperature may also have altered the biochemical properties of the sperm and/or seminal fluid, rendering the sperm more or less competitive. Perez-Velazquez *et al.* (2001) found the sperm quality of shrimps (*Litopenaeus vannamei*) to be affected by exposure to different temperatures. Males exposed to 26°C had a significantly higher sperm count (18.6 million cells) in comparison to those individuals exposed to 29°C (0.1 million cells) (Perez-Velazquez *et al.*, 2001). Furthermore, at 26°C males were shown to have a lower percentage of abnormal sperm (36.7%) compared to individuals exposed to 29°C (99.7% of sperm cells were abnormal). In the common carp (*Cyprinus carpio*) Emri *et al.* (1998) found the ion concentrations in the spermatozoa and seminal plasma to be affected by water temperature. Thus, alteration of the biochemical and physiological properties of sperm and seminal fluid (due to ontogenic heterogeneity) could account for the observed effect of developmental temperature on postcopulatory reproductive success.

In some respects it is not surprising that the ontogenetic environment affects sperm competitive ability as several studies have shown that conditions experienced during development affect sperm biology. For example, Crean & Marshall (2008) found individuals of broadcast spawning tunicate (*Stylea plicata*), reared under high density environments responded with an increase in sperm length, sperm motility and sperm longevity in comparison to those individuals reared at low densities (Crean & Marshall, 2008). Blanckenhorn & Hellriegel (2002), Minoretti *et al.* (2013) and Breckels & Neff (2013) revealed developmental rearing temperature to affect sperm size in dung flies (*S. stercoraria*), the land snail (*A. arbustorum*) and in the guppy (*P. reticulata*) respectively. Vermeulen *et al.* (2009) and Morrow *et al.* (2008) report significant environmental effects on sperm size in the scorpion fly and the fruitfly (*D. melanogaster*) respectively. However, the relationships between sperm size and success in sperm competition is unclear, with a number of studies (Radwan, 1996; LaMunyon & Ward, 1998; Oppliger *et al.*, 2003) reporting a benefit to being large and a number of studies reporting a benefit of being small (Gage & Morrow, 2003; García-González & Simmons, 2007; Voordouw *et al.*, 2008) with the outcome sometimes dependent on the environment in which the competition is played out. For example, in *D. melanogaster* males with longer sperm outcompete males with shorter sperm over fertilization (Miller & Pitnick, 2002) especially when females possess long seminal receptacle lengths.

The observed effect of ontogenic temperature on both the physical and functional properties of sperm is important to evolutionary biology because it provides a mechanism by which intraspecific variation in sperm morphology

is maintained, despite apparent strong stabilizing selection on sperm morphology (Fitzpatrick & Baer, 2011; Immler *et al.*, 2012). It may also represent an ecologically relevant observation, as many insects (ectotherms in general) experience widely fluctuating thermal environments during development. For example, the tobacco hornworm (*Manduca sexta*) experiences a wide range of thermal fluctuations (17°C to 40°C) (Casey, 1976) during larval development which could affect the structure and function of the male and female reproductive traits. In the classic model of sperm competition, *S. stercoraria*, Parker (1971) was always careful to correct for ambient temperature in his calculation of copula duration. However, variation in copula duration and the outcome of sperm competition could be influenced by developmental temperature because dung pats are known to fluctuate by up to 25°C over a short period of time (Penttilä *et al.*, 2013; also see Blanckenhorn *et al.*, 2010). Therefore, developmental temperature may play a key role in influencing the outcome of postcopulatory sexual selection in a large number of species. Thus, it would be interesting to see to what extent fluctuation in ontogenetic environment affects the outcome of sperm competition in other species.

7. General Discussion

7.1 Summary

The primary aim of the investigation was to examine the effects of thermal environment on i) the expression of primary reproductive traits and ii) the consequences of thermal rearing environment on mating behaviour and postcopulatory sexual selection. In order to understand more fully the mechanisms and consequences of the thermal environment on the expression of primary reproductive traits it was necessary to determine:

1. the effect of larval rearing temperature on larval growth and development in general (Chapter 2).
2. the effect of larval rearing temperature on the expression of primary reproductive traits (Chapter 3).
3. when during development larvae were sensitive to changes in the thermal environment, with regard to the expression of sperm length (Chapter 4).
4. the effect of larval rearing temperature on subsequent copulatory behaviour (Chapter 5).
5. the effect of larval rearing temperature on the outcome of sperm competition (Chapter 6).

In many respects Chapter 2 was largely descriptive. Numerous studies (Ward, 1982; Chandrakantha & Mathavan, 1986; Partridge & French, 1996; Arendt & Fairbairn, 2012; Reiskind & Zarrabi, 2012) have shown larval growth and

development to be temperature dependent. The mechanism(s) behind slower growth and development at lower temperatures are most likely slower metabolic processes brought about by slower biochemical processes, which in turn affect a host of factors important for growth including the timing of hormonal synthesis and release (Nijhout & Emlen, 1998; Nijhout, 2003b; Angilletta *et al.*, 2004b; Davidowitz & Nijhout, 2004; Kingsolver *et al.*, 2004a,b; Nijhout *et al.*, 2010). However, it was important to establish the rate of development of *C. maculatus* at different temperatures in order to understand when during development the larvae were sensitive to fluctuations in thermal environment (Chapters 3 & 4). The results from Chapter 2 revealed development from egg-to-adult to take approximately four times longer at 17°C that at 33°C. Additionally, those larvae reared at the lower temperature (17°C) were significantly larger at eclosion than those reared at the higher temperatures, providing support for the well-established Bergmann rule and temperature-size rule (Bergmann, 1847; Angilletta & Dunham, 2003; Angilletta, 2009). However, the data on body size from Chapter 2 adds little to the debate surrounding whether the increase in body size at lower temperatures is a result of an increase in cell size or on an increase in cell number (Partridge *et al.*, 1994; Zamudio *et al.*, 1995; Partridge & French, 1996), nor whether the observed increase in body size at lower temperatures is adaptive (Atkinson, 1994; Huey *et al.*, 1999; Davidowitz *et al.*, 2003; Stillwell *et al.*, 2007). Answering such questions was beyond the scope of this study.

Chapter 3 examined the effect of developmental (larval rearing) and post-eclosion temperature on the expression of primary reproductive traits. In

males the expression of testes size, sperm number and sperm length was affected by larval rearing temperature. In two replicate experiments, absolute testes size was greatest at the lowest larval rearing temperature (17°C), whilst the number of sperm transferred at mating and sperm size were greatest at intermediate temperatures (25°C & 27°C). Post-eclosion temperature experienced by adults (i.e. post eclosion) had no effect on the expression of male primary reproductive traits. These results are valuable because they represent only the fourth ever study to show thermal rearing environment to affect sperm size. Blanckenhorn & Hellriegel (2002) were the first to show sperm length to be affected by larval rearing temperature in the yellow dung fly (*S. stercoraria*). Since then, two further studies have shown developmental temperature to affect subsequent adult sperm size. Minoretti *et al.* (2013) showed that in the land snail (*A. arbustorum*) sperm were shortest when development occurred at the highest temperature, and a similar pattern was found in guppies (*P. reticulata*) (Breckels & Neff, 2013). However, in general few studies have reported plasticity in sperm size in response to developmental conditions. For example, in the Indian meal moth (*P. interpunctella*), nutritional stress and larval density during development affected absolute sperm numbers but not sperm size (Gage & Cook, 1994; Gage, 1995). Gage (1995) framed these responses within the adaptive framework of postcopulatory sexual selection: at high densities males are likely to face a greater risk of sperm competition, thus larger testes and larger ejaculates are likely to enhance male fertilization success. Similar effects of larval density on relative testes size were found in dungflies (*S. stercoraria*) (Stockley & Seal 2001).

In the bivoltine scorpionfly (*P. vulgaris*), seasonality and rearing density affected body size and sperm size. Males of the first annual generation were larger and produced larger sperm than second generation males. Males of the first generation reared at high densities with adequate amounts of food were larger in size but produced smaller sperm in comparison to males that were kept singly and food restricted, suggesting that sperm size is not a simple function of body size in this species (Vermeulen *et al.,* 2009). The difference between 1st and 2nd generation males in sperm length is unlikely to be due to differences in environmental temperature as larvae were reared in the laboratory at 18^{o}C. Morrow *et al*. (2008) undertook a comprehensive analysis of genotype, environment and genotype by environment interactions on sperm length in *D. melanogaster*. They found sperm size to be affected by genotype, the environment (males reared at high larval density produced marginally, but statistically significantly, smaller sperm) and importantly, a genotype x environment (G x E) interaction. This suggests some genotypes are more plastic in their response to environmental change than others and thus, fluctuating environmental conditions in the pre-ejaculatory environment may contribute to the maintenance of intraspecific variation in sperm length.

In contrast to male gametes, several studies have shown plasticity in egg size in relation to larval and adult rearing temperatures (e.g. Azevedo *et al.*, 1996; French *et al.*, 1998; Fischer *et al.*, 2003a). In *D. melanogaster,* laboratory populations reared at low temperatures produced larger eggs than those reared at higher temperatures (Azevedo *et al.*, 1996; French *et al.*, 1998). In the tropical butterfly (*B. anynana*), individuals reared at low temperature

laid larger eggs than females at higher temperature, additionally oviposition temperature yielded similar trends of larger egg size at low temperature suggesting both developmental plasticity and acclimation result in variation in egg size (Fischer *et al.*, 2003a, 2006). The results of the present study lend support to these general trends; females reared at lower temperatures produced larger eggs. This may be adaptive in that the developing offspring are provisioned with greater amounts of resource when development takes place under harsh conditions, although this was not tested in the present study.

Chapter 4 used a thermal-switch protocol to determine when, during larval development, temperature affected the expression of sperm length. This represents the 1st study to use such a protocol to study phenotypic plasticity in sperm length. The data suggests two temperature-sensitive phases in development. With the switch-up protocol, the TSP appears early on in development (instar I) whilst in the switch-down protocol, the TSP occurs around instar III and IV. These two stages are associated with key events in reproductive ontogeny. Further study, with more switch points are required to ascertain exactly, when during development, sperm expression is temperature sensitive. However, these results may be of value to developmental biologists interested in how development proceeds and how development is regulated. In *Drosophila*, developmental regulation of sex-related traits in a complex chain of mechanisms based on gene expression and has elements of autoregulation in which some elements are temperature sensitive (Schütt & Nöthiger, 2000).

Chapters 5 and 6 examine the consequences of variation in larval rearing environment on copulatory behaviour and performance in sperm

competition. Both developmental and post-eclosion temperatures affected copula duration. Copulations that took place at 17°C were the longest. This is in line with several studies that show copulation duration to be negatively related to ambient temperature (Parker, 1970a,b,c; Parker, 1971; Horton *et al.*, 2002; Jiao *et al.*, 2009; Katsuki & Miyatake 2009). The most likely mechanism being a slower metabolic rate at lower temperatures. Males reared at 17°C also copulated for longer. This increase in copulation duration was due to an increase in the 1st phase of copulation (start-to-kick) but not the 2nd stage (kick-to-end). In *C. maculatus,* longer copulation appears to be associated with sperm limitation (see chapter 5). Of interest, the general consensus is that longer copulations allow males to transfer more sperm (e.g. *G. lateralis* (Arnqvist & Danielsson, 1999b); *Adelia bipunctata* (Ransford, 1997; de Jong *et al.*, 1998); *Harmonia axyridis* (Ueno, 1994); *P. vulgaris* (Thornhill & Sauer, 1991) which generally translates to increase fertilization success (Parker & Simmons, 1994). However, developmental stress provides an alternative response; males in poor condition take longer to copulate (see also Wilder & Rypstra, 2007 who report a similar finding in wolf spider (*Hogna helluo*). Of interest, Brown *et al.* (2009) and Gay *et al.* (2011) both report a negative correlation between copulation duration (the male's first copulation) and male longevity. One interpretation of this could be that males in poor condition copulate for longer and are more likely to suffer greater mortality. Another possibility is that reproductive effort trades-off against longevity. Thus the relationship between copula duration, sperm transfer and male fitness may not be straightforward.

Chapter 6 examined the effect of larval rearing temperature on subsequent adult performance in sperm competition. Males reared at 17°C performed worse than those reared at 27°C and 33°C in terms of both sperm offence (P_2) and sperm defence (P_1). This is the first study to show an effect of developmental temperature on the outcome of sperm competition. The poor performance of 17°C males may reflect differences in a number of parameters associated with reproductive physiology such as sperm number, size, physiology (swimming velocity, longevity), volume or composition of seminal fluid, latency to copulation and/or copula duration (e.g. Parker, 1970c; Simmons & Siva-Jothy, 1998; Simmons, 2001; Rurangwa *et al.*, 2004; Hosken *et al.*, 2008; Boschetto *et al.*, 2011), plus their interaction with the female reproductive environment. Further studies to elucidate the mechanisms would be required, although pin-pointing the mechanisms is likely to be difficult as many of these traits are likely to co-vary.

In conclusion, this study has led to new data of relevance to postcopulatory sexual selection and in particular the evolution of primary reproductive traits. Some of these broader implications will now be discussed.

7.2 The role of phenotypic plasticity in the evolution of primary reproductive traits

The evolution of sperm phenotypes has been linked to dimensions of the female reproductive tract (Gomendio & Roldan, 1993; Miller & Pitnick, 2002; Minder *et al.*, 2005), sperm competition (Gomendio & Roldan, 1991; Birkhead & Møller, 1992, 1993; Briskie & Montgomerie, 1992; Roldan *et al.*,

1992), postzygotic reproductive isolation (Karr, 1991) and parental investment (Pitnick & Markow, 1994). However, the environment experienced during ontogeny also induces variation in sperm form (chapters 3 & 4; Blanckenhorn & Hellriegel, 2002; Minoretti *et al.*, 2013; Breckels & Neff, 2013). Below I discuss how phenotypic plasticity might be important to the evolution of sperm phenotypes:

i) Phenotypic plasticity produces new variants upon exposure to novel environmental conditions increasing the likelihood that at least some individuals survive and reproduce in their new environment (Moczek, 2007, 2008; Pfennig *et al.*, 2010).

ii) Phenotypic plasticity may promote the build up of cryptic genetic variation (Moczek, 2008; Pfennig *et al.*, 2010). Essentially, under regular environmental conditions, novel traits are not expressed (due to plasticity) and thus such traits are not exposed to selection. In this way genetic variation for new traits is maintained because it is not selected against. Furthermore, the ability for plasticity to produce new variants only under certain environmental conditions could hint at the existence of a decryptable genetic variant necessary for the genetic accommodation of a novel trait (Moczek, 2007). However, within subsequent generations of a viable population, genetic accommodation of phenotypic variants may be selected to become the more predominantly expressed trait when the genetic variation for that particular trait has gradually increased under environmental novelty (Moczek, 2007; Pfennig *et al.*, 2010).

iii) Developmental plasticity provides new and multiple targets for selection to act (Brakefield *et al.*, 1996). The regulatory interactions that

characterize plasticity are complex (Moczek, 2010; Moczek *et al.*, 2011). External cues (the environment) are transduced into cellular signals by means of hormones, metabolites, receptor molecules, cell membrane permeability, neuronal signals and osmotic changes. "This broad and diverse regulatory dimensionality dramatically increases the potential evolutionary change" (Moczek *et al.*, 2011). Therefore, developmental processes are central for evolution by genetic accommodation as they determine 'when', 'where' and 'what' processes link the genetic and environmental variation in space and time giving rise to genetic accommodation of phenotypic variants (Moczek, 2007, 2008; Moczek *et al.*, 2011).

As West-Eberhard (2005a) pointed out “this view of adaptive evolution is conventional in depicting adaptive evolution as phenotypic change that involves gene frequency change under selection. It departs only slightly, but importantly, from the mutation-selection version of adaptive evolution: although novelties may be induced by mutation they need not be: novelties may be induced by environmental factors". Below, I will discuss the role of plasticity in the evolution of primary reproductive traits such as sperm size evolution but the arguments can be applied to any primary reproductive character.

Sperm phenotypes are generally considered to be canalised (Pitnick *et al.*, 2009) and thus, the ability to produce sperm of a particular (optimal) size under a specific set of environmental conditions, will allow cryptic genetic variation for this trait to build up within the population. Environmental perturbation releases this concealed genetic variation, hidden in regulatory pathways that ultimately produce the sperm phenotype. Under these novel

environmental conditions the new phenotypes and the regulatory pathways that produce these phenotypes represent new and multiple targets for selection (Moczek, 2007). This might simply follow "classic" selection in which a particular phenotype performs best in the new environment or the process may be more complex involving trade-offs. For example, under stressful conditions the best option (in terms of organismal fitness) might be to invest more resources to somatic growth and maintenance at the expense of investment in reproductive traits (see Simmons & Emlen, 2006). Such developmentally plastic responses may then become genetically accommodated. This sequence of events might be particularly relevant to sperm phenotypes because they have to function in both the pre- and post-ejaculatory environments (Morrow *et al.*, 2008). In addition it is becoming increasingly clear that the female reproductive environment, a strong agent of selection on male sperm traits, is also subject to phenotypic plasticity (Berger *et al.*, 2011) and appears to evolve rapidly and divergently (Gage, 2012). Thus, plasticity in the female reproductive environment is likely to change the nature and intensity of selection on male reproductive traits. Therefore, phenotypic plasticity could be very informative in discussions, models and tests of sperm trait evolution.

Phenotypic plasticity may also contribute to the maintenance of genetic variation in primary reproductive traits. Within species, postcopulatory sexual selection might be expected to favour an optimal sperm phenotype (Morrow & Gage, 2001c; Fitzpatrick & Baer, 2011; Immler *et al.*, 2012). In effect, selection is likely to act against sub-optimal sperm types maintaining uniformity in sperm size (Gage & Cook, 1995; Snook, 2005; Birkhead & Pizzari, 2002; see Pattarini *et al.*, 2006). However, within a population it is

common to find variation in sperm size among males (Arnaud *et al.*, 2001; Helfenstein *et al.*, 2008; Morrow *et al.*, 2008, for a review see Pitnick *et al.*, 2009) and within male variation among ejaculates (Harris *et al.*, 2007). Phenotypic plasticity in response to developmental heterogeneity could explain some of this observed intraspecific variation in sperm size (Blanckenhorn & Hellriegel, 2002; Minoretti *et al.*, 2013; Breckels & Neff, 2013). Environmental heterogeneity during development is likely to be widespread in nature (Blanckenhorn, 2000; Blanckenhorn & Henseler, 2005; Oostra *et al.*, 2011; Rodríguez, 2012; Breckels & Neff, 2013) and is likely to result in selection for different trade-offs (Blanckenhorn & Hellriegel, 2002; Simmons & Emlen, 2006; Berger *et al.*, 2011). Thus, and hypothetically, it is possible that larvae exposed to extreme environments during development face a trade-off between investment in soma versus investment in germ line. Such that under extreme conditions more resources are likely to be allocated to the maintenance of soma, leaving fewer resources to be allocated to reproduction. As a consequence, these individuals may produce fewer or smaller sperm. Although, this may appear to be non-adaptive within the context of postcopulatory sexual selection (chapter 6 and below) it is possible that it is the best solution to the individual: the choice may be to survive the extreme conditions with poor ejaculatory traits or die prior to reproducing (but with larger sperm). Clearly from an evolutionary perspective, the former strategy is better. This hypothetical example illustrates how heterogeneity in the developmental, pre-ejaculatory environment could result in G x E trade-offs, which ultimately results in variation in sperm phenotypes (see also Morrow *et al.*, 2008).

Genotype x environment interactions across heterogeneous environments can help maintain genetic variation (i.e. the fitness of genotypes vary across environments, such that selection favours different genotypes in different environments) which can be measured by the different patterns of norms of reaction (genotypes ranked by fitness across environments) (Scheiner, 1993; Rodríguez, 2012). Variation in G x E interactions can largely be explained by the grain-size of the environment experienced during ontogeny, in which grain-size refers to the scale at which environmental heterogeneity occurs in relation to generation time (Rodríguez, 2012); fine-grained refers to a situation in which an individual from one generation encounters many environments, whilst a coarse-grained environment refers to an individual of one generation encountering a single environment type (Rodríguez, 2012). Under finer-grained environments it has been suggested that heterogeneous selection can generate novel phenotypes via alterations to developmental pathways or trade-offs in physiological/biochemical pathways (i.e. plasticity). However, in fine-grained environments selection favours phenotypic plasticity and thus erodes the genetic variation in reaction norms, because, in the face of predictable environmental heterogeneity, a single optimal, norm of reaction will be favoured. In contrast, under coarse-grained environments, G x E interactions (i.e. the genetic variation in phenotypic plasticity) are predicted to be larger because environmental heterogeneity is encountered less frequently. Hence, selection for a single norm of reaction is lessened. Here, selection loses the ability to shape norms of reaction, resulting in greater genetic variation in the norms of reaction (Rodríguez, 2012).

7.3 Mechanisms underlying phenotypic plasticity in response to environmental temperature

Temperature has been shown to affect membrane fluidity via its effects on the motion of the fatty acyl chains of glycerophospholipids and ectotherms have the capacity to alter the lipid composition of membranes in response to changes in external temperature both in the short term (acclimation) and long term (evolutionary adaptation) (Cooper *et al.*, 2012b). For example, populations of *D. melanogaster* reared under heterogeneous, thermal conditions for three years evolved greater cellular membrane plasticity than populations reared under constant temperature environments. Flies reared under heterogeneous environments had greater capacity to acclimate the phosphatidylethanolamine : phosphatidylcholine (PE:PC) ratio compared to flies evolved under constant environmental conditions (Cooper *et al.*, 2012b). Thus, it is possible that the *C. maculatus* larvae reared under thermal extremes in this study, either experience a compromised cell membrane function and/or undergo cell membrane acclimation (as described above), that subsequently alters developmental pathways, producing the observed phenotypic plasticity in sperm size.

During very early development, the fate of the gonads can be influenced by environmental variation through modifications to molecular pathways (Navarro-Martín *et al.*, 2011). In the European sea bass *Dicentrarchus labrax*, juvenile males have approximately twice as much DNA methylation in the promoter of gonadal aromatase *cyp19a* than females. DNA methylation (the addition of a methyl group to cytosine or adenine) can alter gene expression in cells, such that genes with high levels of methylation in

their promoter region are transcriptionally silent. Aromatase is fundamental to sexual differentiation in this species because it induces androgens to synthesize estrogens. Females exposed to high temperatures during early development experience greater levels of methylation in the *cyp19a* promoter inducing masculinization. Thus, the molecular mechanisms that control gonad morphogenesis in the sea bass are both present early during embryogenesis and temperature sensitive (Navarro-Martín *et al.*, 2011). Similar temperature-sensitive mechanisms could alter the developmental trajectories of the male gonads in *C. maculatus*.

In *Drosophila*, the control of sexual development (both gametic and sex-specific somatic) is a complex process (Schütt & Nöthiger, 2000). The primary sexual signal for sexual differentiation of the soma is the ratio of X chromosomes to autosomes (X:A), which is 1.0 in females and 0.5 in males. The X:A signal is interpreted by maternal genes which act to regulate the key gene, *sex-lethal*. *Sex-lethal* is activated in females and not in males. The *sex-lethal* protein autoregulates *sex-lethal* activity and controls the expression of downstream genes that control regulatory cascades that ultimately produce male or female phenotypes. One of the target genes of *sex-lethal* is *transformer* (*tra*). The *TRA* protein promotes female-specific splicing of the *double-sex* gene (*dsx*). In the absence of *TRA* the transcripts of *dsx* are male specific. *DSXm* (the male transcript) represses genes required for female differentiation. *DSXf* does the opposite. Of interest, in *Drosophila*, *tra2* is temperature sensitive (Belote & Baker, 1982), thus XX individuals raised at the permissive temperature of 16°C develop into females, whereas XX individuals raised at 29°C become sterile males (Belote & Baker, 1982; Schütt

& Nöthiger, 2000). In *Drosophila*, sex determination of the germ line is less well understood, although their differentiation is thought to be via inductive signals from the soma. Thus, the temperature sensitive *tra2* gene affects sex-specific somatic development which could subsequently affect germ-line development and consequently the ability of the individual to produce 'normal' gametes. Schütt & Nöthiger (2000) point out that *sex-lethal* does not have a sex-determining function in any other taxa studied so far, indicating that sex-determining cascades appear to evolve rapidly. Thus, although temperature-sensitive genes play a role in sexual differentiation in *Drosophila* it is not clear if they play a role in other taxa. Therefore, whether *C. maculatus* has temperature sensitive genes remains speculative.

7.4 Is developmental plasticity of sperm size adaptive?

At present it is not known if sperm size plasticity in response to developmental temperature is adaptive or not. The demonstration of poor performance in sperm competition experiments held at 27°C and 'carried out' in the reproductive tract of females reared at 27°C simply tell us that these sperm do not function well under these conditions. To test for an adaptive function, it would be necessary to determine if the sperm of males that developed at 17°C and 33°C functioned better (or not) when placed in competition against the sperm of 27°C males when the competition either took place at 17°C and 33°C or within the reproductive environments of females reared at 17°C and 33°C. It could be that the smaller sperm of the 17°C or 33°C (reared) males have a functional advantage if the ambient environment is close to one of these rearing temperatures. In a similar vein, the sperm of 17°C or 33°C males

might function best in the reproductive tract of females that developed at 17°C or 33°C.

Of interest, Berger *et al.* (2011) have recently demonstrated phenotypic plasticity in response to developmental temperature in female reproductive tract in yellow dung flies (*S. stercoraria*). The female reproductive environment sets the rules by which sperm competition is played out (Eberhard, 1996; Wilson *et al.*, 1997) and it is well established that male and female reproductive traits show correlated evolution (Dybas & Dybas, 1981; Briskie & Montgomerie, 1992; Presgraves *et al.*, 1999; Morrow & Gage, 2000; Miller & Pitnick, 2002; Minder *et al.*, 2005; Rugman-Jones & Eady, 2008; Gage, 2012). Thus it is possible that developmental temperature affects female reproductive anatomy and physiology, such that males producing alternative sperm phenotypes (through plastic responses to environmental change) may be selected for (via enhanced success in postcopulatory sexual selection), when sperm competition takes place within the 'novel' reproductive environments.

Alternatively though, the influence of developmental temperature on the expression of reproductive traits could also be due to non-adaptive constraints imposed on the underlying physiological processes (Berger *et al.*, 2012). Berger *et al.* (2012) showed that temperature was the most contraining ecological factor influencing the reproductive physiology of the butterfly (*Leptidea synpasis*). They argued that temperature constrained the maintenance of various functional aspects of life-history via disproportionately partitioning bodily resources differently among the sexes.

7.5 Conclusions and future directions

The results presented in this thesis indicate sperm size and function (competitive success) to be, in part, a consequence of developmental temperature. This phenotypic plasticity most likely arises as a product of changes to the underlying regulatory processes that control trait expression. Several aspects and ideas can be investigated in the future as a result of this study:

- The long term effects of developmental temperature on sperm traits would reveal whether or not sperm phenotypes evolve (become genetically accommodated) in response to natural rather than sexual selection. This experiment will help understand the notion that whether developmentally plastic sperm length of this species can eventually become genetically accommodated after many generations of directional selection.
- Since larval rearing temperature affects the outcome of postcopulatory sexual selection at 27°C (chapter 6), the obvious next stage would be to examine the adaptive nature of phenotypic plasticity in sperm traits by measuring the outcome of postcopulatory sexual selection (P_1 and P_2) at different environmental temperatures. Thus, do the sperm of males reared at 17°C or 33°C perform best/better in sperm competition when the competition takes place at 17°C or 33°C? In a similar vein, it would be informative to measure P_1 and P_2 of 17°C or 33°C males where sperm competition takes place within the reproductive tract of females reared at these temperatures.
- The effect of developmental temperature on the plasticity of female reproductive tract morphology? Females have rarely been investigated for

changes to the morphology of their reproductive tract induced by developmental environment (e.g. temperature) and developmental heterogeneity is commonplace. Hribar (1996) showed that larval rearing temperature affected the expression of male genitalia in a species of mosquito (*A. albimanus*). Experimentally inducing changes to the reproductive morphology of females could result in rapid correlated changes to the males. Thus, developmental temperature could drive the evolution of both male and female genitalia and this could influence the mechanisms of sexual selection.

- How thermal switch protocols affect the outcome of postcopulatory sexual selection. Temperature switching during larval development affected sperm size. The next step would be to determine the fitness (success in sperm competition) of males that experience a thermal switch during development.
- Larval rearing environment has an effect on sperm size, but little is known about the effect of thermal rearing environment on the expression of other primary reproductive traits, such as the morphology of the intromittent organ and/or the chemical nature of the seminal fluids. Therefore, a sensible next step would be to quantify the role of developmental plasticity on aedeagal morphology, (possibly using geometrics and morphometrics) and/or the chemical composition of the ejaculate.
- To study the reaction norms for sperm length in other closely related bruchid species (to quantify the total variation for sperm size across environments) in response to fluctuating larval rearing temperatures. This will reveal the nature of the types of reaction norms for sperm size and

evaluated for other reproductive traits (e.g. testis size). The investigation is likely to throw light on genotype x environment (G x E) relationships and if phenotypic plasticity facilitates or constraints the evolution of sperm form and function in the Bruchids.

Postcopulatory sexual selection has emerged as a key front runner as the selective agent driving the rapid and divergent evolution of primary reproductive traits (Eberhard, 1996; Simmons, 2001; Birkhead *et al.*, 2009; Leonard & Córdoba-Aguilar, 2010; Gage, 2012). However, studies regarding the role of natural selection in primary reproductive tract evolution are generally scarce. Here, I have shown that phenotypic/developmental plasticity in primary reproductive traits in response to environmental conditions that are usually considered in a naturally selected context (i.e. temperature) can affect sperm structure and function. Given the renewed interest in the role of plasticity in the evolutionary process (e.g. Swanson & Vacquier, 2002; Bradshaw & Holzapfel, 2006; Moczek, 2007, 2008; Turner & Hoekstra, 2008; Beldade *et al.*, 2011; Nevo, 2011; Grazer & Martin, 2012a,b; Caro *et al.*, 2013) it is hoped that this thesis will stimulate more work on the role of natural selection and in particular the interplay between natural and sexual selection on the evolution of primary reproductive traits.

References

Adams, D. C. & Church, J. O. (2007). Amphibians do not follow Bergmann's rule. *Evolution.* 62, 413–420.

A Dictionary of Science, 5th Edition. (2005) (Eds. Daintith, J.; Martin, E.) New York, New York, USA: Oxford University Press.

Alpatov, W. W. (1930). Phenotypical variation in body and cell size of *Drosophila melanogaster*. *The Biological Bulletin.* 58, 85–103.

Amitin, E. G. & Pitnick, S. (2007). Influence of developmental environment on male- and female-mediated sperm precedence in *Drosophila melanogaster*. *Journal of Evolutionary Biology*. 20, 381–391.

Ancel, L. W. (1999). A quantitative model of the Simpson-Baldwin effect. *Journal of Theoretical Biology.* 196, 197–209.

Ancel, L. W. (2000). Undermining the Baldwin expediting effect: does phenotypic plasticity accelerate evolution? *Theoretical Population Biology*. 58, 307–319.

Andersson, M. (1982). Female choice selects for extreme tail length in a widowbird. *Nature*. 299, 818–820.

Andersson, M. (1994). Sexual Selection. Princeton, Princeton University Press.

Andersson, M. & Simmons, L. W. (2006). Sexual selection and mate choice. *Trends in Ecology & Evolution.* 21, 296–302.

Andrade, C. A. C., Hatadani, L. M. & Klaczko, L. B. (2005). Phenotypic plasticity of the aedeagus of *Drosophila mediopunctata*: effect of the temperature. *Journal of Thermal Biology*. 30, 518–523.

Andrés, J. A. & Rivera, A. C. (2000). Copulation duration and fertilization success in a damselfly: an example of cryptic female choice? *Animal Behaviour*. 59, 695–703.

Angilletta, M. J., Jr. (2009). *Thermal Adaptation: a theoretical and empirical synthesis.* Oxford University Press, Oxford, UK.

Angilletta, M. J., Jr. & Dunham, A. E. (2003). The temperature-size rule in ectotherms: simple evolutionary explanations may not be general. *The American Naturalist.* 162, 332–342.

Angilletta, M. J., Jr., Niewiarowski, P. H. & Navas, C. A. (2002). The evolution of thermal physiology in ectotherms. *Journal of Thermal Biology*. 27, 249–268.

Angilletta, M. J., Jr., Wilson, R. S., Navas, C. A. & James, R. S. (2003). Tradeoffs and the evolution of thermal reaction norms. *Trends in Ecology & Evolution.* 18, 234–240.

Angilletta, M. J., Jr., Niewiarowski, P. H., Dunham, A. E., Leaché, A. D. & Porter, W. P. (2004a). Bergmann's clines in Ectotherms: Illustrating a life-history perspective with Sceloporine lizards. *The American Naturalist.* 164, E168–E183.

Angilletta, M. J., Jr., Steury, T. D. & Sears, M. W. (2004b). Temperature, growth rate and body size in ectotherms: Fitting pieces of a life-history puzzle. *Integrative & Comparative Biology*. 44, 498–509.

Arendt, J. & Fairbairn, D. (2012). Reproductive allometry does not explain the temperature-size rule in water striders (*Aquarius remigis*). *Evolutionary Ecology.* 26, 745–757.

Arnaud, L., Haubruge, E. & Gage, M. J. G. (2008). Sperm size and number variation in the red flour beetle. *Zoological Journal of the Linnean Society*. 133, 369–375.

Arnqvist, G. & Danielsson, I. (1999a). Copulatory behaviour, genital morphology and male fertilization success in water striders. *Evolution.* 53, 147–156.

Arnqvist, G. & Danielsson, I. (1999b). Postmating sexual selection: the effects of male body size and recovery period on paternity and egg production rate in a water strider. *Behavioural Ecology*. 10, 358–365.

Arnqvist, G. & Nilsson, T. (2000). The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*. 60, 145–164.

Arnqvist, G. & Rowe, L. (2005). *Sexual conflict*. Princeton University Press.

Atkinson, D. (1994). Temperature and organism size- a biological law for ectotherms. *Advances in Ecological Research.* 25, 1–58.

Atkinson, D. & Silby, R. M. (1997). Why are organisms usually bigger in colder environments? Making sense of a life-history puzzle. *Trends in Ecology & Evolution.* 12, 235–239.

Atkinson, D., Morley, S. A. & Hughes, R. N. (2006). From cells to colonies: at what levels of body organization does the ‘temperature-size rule’ apply. *Evolution & Development.* 8, 202–214.

Austad, S. N. (1984). Evolution of sperm priority patterns in spiders. In *Sperm competition* and *the evolution of Animal Mating Systems*. Smith, R. L. (ed). pp. 233–249. Academic Press: London.

Azevedo, R. B. R., French, V. & Partridge, L. (1996). Thermal evolution of egg size in *Drosophila melanogaster*. *Evolution.* 50, 2338–2345.

Azevedo, R. B. R., French, V. & Partridge, L. (2002). Temperature modulates epidermal cell size in *Drosophila melanogaster*. *Journal of Insect Physiology.* 48, 231–237.

Baccetti, B. (1986). Evolutionary trends in sperm structure. *Comparative Biochemistry & Physiology Part A.* 85, 29–36.

Barata, C., Baird, D. J. & Soares, A. M. V. M. (2001). Phenotypic plasticity in *Daphnia magna*: variable maturation instar as an adaptive response to predation pressure. *Oecologia.* 129, 220–227.

Barker, J. F. & Herman, W. S. (1976). Effect of photoperiod and temperature on reproduction of the monarch butterfly, *Danaus plexippus. Journal of Insect Physiology.* 22, 1565–1568.

Basso, A. L., Forneris, N. S., Filiberti, A., Argaraña, C. E., Rabossi, A. & Quesada-Allué, L. A. (2011). Metamorphosis and gonad maturation in the horn fly *Haematobia irritans*. *Journal of Insect Science*. 11, 1–10.

Beatty, R. A. (1970). The genetics of the mammalian gamete. *Biological Reviews.* 45, 73–119.

Beck, C. W. & Blumer, L. S. (2011). A Handbook on Bean Beetles, *Callosobruchus maculatus*. (http://www.beanbeetles.org/handbook/handbook.pdf).

Beldade, P., Mateus, A. R. A. & Keller, R. A. (2011). Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology*. 20, 1347–1363.

Belote, J. M. & Baker, B. S. (1982). Sex determination in *Drosophila melanogaster*: Analysis of transformer-2, a sex-transforming locus. *Proceedings of the National Academy of Sciences USA.* 79, 1568–1572.

Belote, J. M. & Baker, B. S. (1987). Sexual behaviour: Its genetic control during development and adulthood in *Drosophila melanogaster. Proceedings of the National Academy of Sciences USA.* 84, 8026–8030.

Bennett, A. F. (1987). The accomplishments of ecological physiology. In *New Directions in Ecological Physiology* (Eds. Feder, M. E., Bennett, A. F., Huey, R. B. & Burggren, W.) pp. 1–8. New York, Cambridge University Press.

Berger, D., Bauerfeind, S. S., Blanckenhorn, W. U. & Schäfer, M. A. (2011). High temperatures reveal cryptic genetic variation in a polymorphic female sperm storage organ. *Evolution*. 65, 2830-2842.

Berger, D., Olofsson, M., Gotthard, K., Wiklund, C. & Friberg, M. (2012). Ecological constraints on female fitness in a phytophagous insect. *The American Naturalist*. 180, 464–480.

Bergmann, C. (1847). Über die verhältnisse der wärmeökonomie der thiere zu ihrer grösse. *Göttinger Studien.* 3, 595–708.

Bhat, S. G. & Babu, P. (1987). *Wingless* mutation in *Drosophila melanogaster*. *Journal of Biosciences.* 12, 1–11.

Birkhead, T. R. (1998). Cryptic female choice: criteria for establishing female sperm choice. *Evolution*. 52, 1212–1218.

Birkhead, T. R. & Fletcher, F. (1995). Male phenotype and ejaculate quality in the zebra finch *Taeniopygia guttata*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 262, 329–334.

Birkhead, T. R. & Møller, A. P. (1992). Sperm competition in birds. Evolutionary causes and Consequences. Academic Press, London.

Birkhead, T. R. & Møller, A. P. (1998). *Sperm Competition and Sexual Selection*, Academic Press.

Birkhead, T. R., Fletcher, F., Pellatt, E. J. & Staples, A. (1995). Ejaculate quality and the success of extra-pair copulations in the zebra finch. *Nature*. 377, 422–423.

Birkhead, T. R. & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews Genetics*. 3, 262–273.

Blanckenhorn, W. U. (1997). Altitudinal life history variation in the dung flies *Scathophaga stercoraria* and *Sepsis cynipsea*. *Oecologia*. 109, 342–352.

Blanckenhorn, W. U. (1999). Different growth responses to temperature and resource limitation in three fly species with similar life-histories. *Evolutionary Ecology.* 13, 395–409.

Blanckenhorn, W. U. (2000). Temperature effects on egg size and their fitness consequences in the yellow dung fly *Scathophaga stercoraria. Evolutionary Ecology.* 14, 627–643.

Blanckenhorn, W. U. & Hellriegel, B. (2002). Against Bergmann's rule: fly sperm size increases with temperature. *Ecology Letters.* 5, 7–10.

Blanckenhorn, W. U. & Henseler, C. (2005). Temperature-dependent ovariole and testis maturation in the yellow dung fly. *Entomologia Experimentalis et Applicata.* 116, 159–165.

Blanckenhorn, W. U., Dixon, A. F., Fairbairn, D. J., Foellmer, M. W., Gibert, P., van der Linde, K., Meier, R., Nylin, S., Pitnick, S., Schoff, C., Signorelli, M., Teder, T. & Wiklund, C. (2007). Proximate causes of Rensch's rule: Does sexual size dimorphism in Arthropods result from sex differences in development time? *The American Naturalist*. 169, 245–257.

Blanckenhorn, W. U., Pemberton, A. J., Bussière, L. F., Roembke, J. & Floate, K. D. (2010). A review of the natural history and laboratory culture methods for the yellow dung fly, *Scathophaga stercoraria*. *Journal of Insect Science*. 10, 1–17.

Blouin, M. S. & Brown, S. T. (2000). Effects of temperature induced variation in anuran larval growth rate on head width and leg length at metamorphosis. *Oecologia*. 125, 358–361.

Boorman, E. & Parker, G. A. (1976). Sperm (ejaculate) competition in *Drosophila melanogaster* and the reproductive value of females to males in relation to female age and mating status. *Ecological Entomology*. 1, 145–155.

Booth, D. T. (2006). Influence of Incubation Temperature on Hatchling Phenotype in Reptiles. *Physiological & Biochemical Zoology*. 79, 274–281.

Boschetto, C., Gasparini, C. & Pilastro, A. (2011). Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behavioural Ecology & Sociobiology*. 65, 813–821.

Bounds, C., Wilson, R. & Marshall, D. J. (2010). Why do colder mothers produce larger eggs? An optimality approach. *The Journal of Experimental Biology.* 213, 3796–3801.

Bradshaw, W. E. & Holzapfel, C. M. (2006). Evolutionary response to rapid climate change. *Science.* 312, 1477–1478.

Brakefield, P. M. (2006). Evo-devo and constraints on selection. *Trends in Ecology & Evolution*. 21, 362–368.

Brakefield, P. M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P. J., Monteiro, A., French, V. & Carroll, S. B. (1996). Development, plasticity and evolution of butterfly eyespot patterns. *Nature*. 384, 236–242.

Brazner, J. C. & Etges, W. J. (1993). Pre-mating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. II. Effects of larval substrates on time to copulation, mate choice and mating propensity. *Evolutionary Ecology*. 7, 605–624.

Breckels, R. D. & Neff, B. D. (2013). The effects of elevated temperature on the sexual traits, immunology and survivorship of a tropical ectotherm. *The Journal of Experimental Biology*. 216, 2658–2664.

Briskie, J. V. & Montgomerie, R. (1992). Sperm size and sperm competition in birds. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 247, 89–95.

Brown, D. V. (2001). Postcopulatory sexual selection in *Callosobruchus maculatus*. Ph.D. Thesis, University of Sunderland, UK.

Brown, E. A., Gay, L., Vasudev, R., Tregenza, T., Eady, P. E. & Hosken, D. J. (2009). Negative phenotypic and genetic associations between

copulation duration and longevity in male seed beetles. *Heredity*. 103, 340–345.

Bull, J. J. (1983). *Evolution of sex determining mechanisms*. Benjamin/Cummings, Menlo Park, California, USA.

Bull, J. J. (1987). Temperature-sensitive periods of sex determination in lizard: similarities with turtles and crocodilians. *Journal of Experimental Zoology*. 241, 143–148.

Burley, N., Krantzberg, G. & Radman, P. (1982). Influence of colour-banding on the conspecific preferences of zebra finches. *Animal Behaviour*. 30, 444–455.

Caro, S. P., Schaper, S. V., Hut, R. A., Ball, G. F. & Visser, M. E. (2013). The case of the missing mechanism: how does temperature influence seasonal timing in endotherms? *PLoS Biology*. 11, 1–8.

Carroll, S. P. (1991). The adaptive significance of mate guarding in the soapberry bug, *Jadera haematoloma* (Hemiptera: Rhopalidae). *Journal of Insect Behaviour*. 4, 509–530.

Casey, T. M. (1976). Activity patterns, body temperature and thermal ecology in two desert caterpillars (Lepidoptera: Sphingidae). *Ecology*. 57, 485–497.

Casselman, S. J., Schulte-Hostedde, A. I. & Montgomerie, R. (2006). Sperm quality influences male fertilization success in walleye (*Sander vitreus*). *Canadian Journal of Fisheries & Aquatic Sciences*. 63, 2119–2125.

Cepeda-Palacios, R., Monroy, A., Mendoza, M. A. & Scholl, P. J. (2001). Testicular maturation in the sheep bot fly *Oestrus ovis*. *Medical & Veterinary Entomology*. 15, 275–280.

Chandrakantha, J. & Mathavan, S. (1986). Changes in developmental rates and biomass energy in *Callosobruchus maculatus (F)* (Coleoptera: Bruchidae) reared on different foods and temperatures. *Journal of Stored Product Research.* 22, 71–75.

Chandrakantha, J., Muthukrishnan, J. & Mathavan, S. (1987). Effect of temperature and host seed species on the fecundity of *Callosobruchus maculatus* (F.). *Proceedings of the Indian Academy of Sciences*. 96, 221–227.

Chapman, R. F. (1998). *The Insects: Structure and Function*. Cambridge University press, Cambridge.

Chapman, T., Arnqvist, G., Bangham, J. & Rowe, L. (2003). Sexual conflict. *Trends in Ecology & Evolution.* 18, 41–47.

Charnov, E. L. & Bull, J. J. (1977). When is sex environmentally determined? *Nature*. 266, 828–830.

Chen, Z. Q., Jiao, X. G., Wu, J., Chen, J. & Liu, F. X. (2010). Effects of copulation temperature on female reproductive output and longevity in the wolf spider *Pardosa astrigera* (Araneae: Lycosidae). *Journal of Thermal Biology*. 35, 125–128.

Ciofi, C. & Swingland, I. R. (1997). Environmental sex determination in reptiles. *Applied Animal Behaviour Science.* 51, 251–265.

Clark, A. G., Begun, D. J. & Prout, T. (1999). Female x male interactions in *Drosophila* sperm competition. *Science*. 283, 217–220.

Clutton-Brock, T. H., Guinness, F. E. & Albon, S. D. (1982). *Red deer: behaviour and ecology of two sexes*. University of Chicago Press, Chicago.

Cook, P. A. & Gage, M. J. G. (1995). Effects of risk of sperm competition on the numbers of eupyrene and apyrene sperm ejaculated by the moth *Plodia interpunctella* (Lepidoptera: Pyralidae). *Behavioural Ecology & Sociobiology*. 36, 261–268.

Cooper, B. S., Tharp II, J. M., Jernberg, I. I. & Angilletta, M. J., Jr. (2012a). Developmental plasticity of thermal tolerances in temperate and subtropical populations of *Drosophila melanogaster*. *Journal of Thermal Biology*. 37, 211–216.

Cooper, B. S., Hammad, L. A., Fisher, N. P., Karty, J. A. & Montooth, K. L. (2012b). In a variable thermal environment selection favors greater plasticity of cell membranes in *Drosophila melanogaster*. *Evolution*. 66, 1976–1984.

Córdoba-Aguilar, A. (2009). Seasonal variation in genital and body size, sperm displacement ability, female mating rate, and male harassment in two Calopterygid damselflies (Odonata: Calopterygidae). *Biological Journal of the Linnean Society*. 96, 815–829.

Crawley, M. J. (2002). *Statistical Computing: An Introduction to Data Analysis Using S-PLUS*. John Wiley & Sons.

Crean, A. J. & Marshall, D. J. (2008). Gamete plasticity in a broadcast spawning marine invertebrate. *Proceedings of the National Academy of Sciences USA*. 105, 13508–13513.

Crews, D., Bergeron, J. M., Bull, J. J., Flores, D., Tousignant, A., Skipper, J. K. & Wibbels, T. (1994). Temperature-dependent sex determination in reptiles: proximate mechanisms, ultimate outcomes, and practical applications. *Developmental Genetics*. 15, 297–312.

Crispo, E. (2008). Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. *Journal of Evolutionary Biology*. 21, 1460–1469.

Crudgington H. S. & Siva-Jothy, M. T. (2000). Genital damage, kicking and early death. *Nature*. 407, 855–856.

Daufresne, M., Lengfellner, K. & Sommer, U. (2009). Global warming benefits the small in aquatic ecosystems. *Proceedings of the National Academy of Sciences USA.* 106, 12788–12793.

Davidowitz, G., D'Amico, L. J. & Nijhout, H. F. (2003). Critical weight in the development of insect body. *Evolution & Development.* 5, 188–197.

Davidowitz, G., D'Amico, L. J. & Nijhout, H. F. (2004). The effects of environmental variation on a mechanism that controls insect body size. *Evolutionary Ecology Research.* 6, 49–62.

Davidowitz, G. & Nijhout, H. F. (2004). The physiological basis of reaction norms: The interaction among growth rate, the duration of growth and body size. *Intergrative & Comparative Biology.* 44, 443–449.

de Jong, G. (2005). Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. *New Phytologist*. 166, 101–118.

De Moed, G. H., de Jong, G. & Scharloo, W. (1997). Environmental effects on body size variation in *Drosophila melanogaster* and its cellular basis. *Genetical Research.* 70, 35–43.

Deeming, D. C. & Ferguson, M. W. J. (1989a). The mechanism of temperature dependent sex determination in Crocodilians: A Hypothesis. *American Zoologist.* 29, 973–985.

Deeming, D. C. & Ferguson, M. W. J. (1989b). In the heat of the nest. *New Scientist*. 1657, 33–38.

Deeming, D. C. & Ferguson, M. W. J. (1991). Physiological effects of incubation temperature on embryonic development in reptiles and birds. In *Egg incubation: Its effects on embryonic development in birds and reptiles* (Eds. Deeming, D. C & Ferguson, M. W. J.) pp. 147—171. Cambridge University Press.

Deeming, D. C., Ferguson, M. W. J., Mittwoch, U., Wolf, U., Dorizzi, M., Zaborski, P. & Sharma, H. (1988). Environmental regulation of sex determination in reptiles. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences.* 322, 19–39.

Demerec, M. (1994). *The Biology of Drosophila*. John Wiley & Sons, reprinted by Cold Spring Harbor Laboratory Press.

Desvages, G., Girondot, M. & Pieau, C. (1993). Sensitive Stages for the Effects of Temperature on Gonadal Aromatase Activity in Embryos of the Marine Turtle *Dermochelys coriacea*. *General & Comparative Endocrinology*. 92, 54–61.

Devereau, A. D., Gudrups, I., Appleby, J. H. & Credland, P. F. (2003). Automatic, rapid screening of seed resistance in cowpea, Vigna unguiculata (L.) Walpers, to the seed beetle *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) using acoustic monitoring. *Journal of stored Product Research*. 39, 117–129.

Dewitt, T. J. & Scheiner, S. M. (2004). Phenotypic variation from single genotypes: a primer. In *Phenotypic plasticity: Functional and conceptual Approaches.* (Eds. DeWitt, T. J. & Schenier, S. M.) pp. 1–9. Oxford University Press, Oxford.

Dybas, L. K. & Dybas, H. S. (1981). Coadaptation and Taxonomic differentiation of sperm and spermathecae in Featherwing beetles. *Evolution.* 35, 168–174.

Dziminski, M. A., Dale Roberts, J., Beveridge, M. & Simmons, L. W. (2009). Sperm competitiveness in frogs: slow and steady wins the race. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 276, 3955–3961.

Eady, P. E. (1991). Sperm competition in *Callosobruchis maculatus* (Coleoptera: Bruchidae): a comparison of two methods used to estimate paternity. *Ecological Entomology.* 16, 45–53.

Eady, P. E. (1994a). Intraspecific variation in sperm precedence in *Callosobruchus maculatus. Ecological Entomology.* 16, 45–53.

Eady, P. E. (1994b). Sperm transfer and storage in relation to sperm competition in *Callosobruchus maculatus. Behavioural Ecology & Sociobiology.* 35, 123–129.

Eady, P. E. (1995). Why do male *Callosobruchus maculatus* beetles inseminate so many sperm? *Behavioural Ecology & Sociobiology.* 36, 25–32.

Eady, P. E., Rugman-Jones, P. & Brown, D. V. (2004). Prior oviposition female receptivity and last-male sperm precedence in the cosmopolitan pest *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Animal Behaviour.* 67, 559–565.

Eady, P. E., Hamilton, L. & Lyons, R. E. (2007). Copulation, genital damage and early death *in Callosobruchus maculatus*. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 274, 247–252.

Eberhard, W. G. (1985). *Sexual Selection and Animal Genitalia*. Cambridge, Mass: Harvard University Press.

Eberhard, W. G. (1996). *Female control: sexual selection by cryptic female choice*. Princeton University Press, Princeton, NJ.

Eberhard, W. G. (2010). Genitalic evolution: theories and data updated. In *Evolution of Primary Sexual Characters in Animals* (Eds. Leonard, J. L. & Cordoba-Aguilar, A.) pp. 40–78, Oxford University Press.

Eberhard, W. G. (2011). Experiments with genitalia: a commentary. *Trends in Ecology & Evolution*. 26, 17–21.

Edgar, B. A. (2006). How flies get their size: genetics meets physiology. *Nature Reviews Genetics*. 7, 907–916.

Edvardsson, M. & Canal, D. (2006). The effects of copulation duration in the bruchid beetle *Callosobruchus maculatus. Behavioural Ecology*. 17, 430–434.

Edward, D. A. & Chapman, T. (2012). Sex-specific effects of developmental environment on reproductive trait expression in *Drosophila melanogaster*. *Ecology & Evolution*. 2, 1362–1370.

Emri, M., Marian, T., Tron, L., Balkay, L. & Krasznai, Z. (1998). Temperature adaptation changes ion concentrations in spermatozoa and seminal plasma of common carp without affecting sperm motility. *Aquaculture*. 167, 85–94.

Endler, J. A. (1980). Natural selection on color patterns in *Poecilia reticulata*. *Evolution.* 34, 76–91.

Espinosa-Soto, C., Martin, O. C. & Wagner, A. (2011). Phenotypic plasticity can facilitate adaptive evolution in gene regulatory circuits. *BMC Evolutionary Biology*. 11, 1–14.

Feast, M., Noble, R. C., Speake, B. K. & Ferguson, M. W. J. (1998). The effect of temporary reductions in incubation temperature on growth characteristics and lipid utilisation in the chick embryo. *Journal of Anatomy*. 193, 383–390.

Fischer, K., Bauerfeind, S. S. & Fiedler, K. (2006). Temperature-mediated plasticity in egg and body size in egg size-selected lines of a butterfly. *Journal of Thermal Biology*. 31, 347–354.

Fischer, K., Eenhoorn, E., Bot, A. N., Brakefield, P. M. & Zwaan, B. J. (2003a). Cooler butterflies lay larger eggs: developmental plasticity versus acclimation. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 270, 2051–2056.

Fischer, K., Brakefield, P. M. & Zwaan, B. J. (2003b). Plasticity in butterfly egg size: why larger offspring at lower temperatures? *Ecology*. 84, 3138–3147.

Fitzpatrick, B. M. (2012). Underappreciated consequences of phenotypic plasticity for ecological speciation. *International Journal of Ecology*. 2012, 1–12.

Fitzpatrick, J. L. & Baer, B. (2011). Polyandry reduces sperm length variation in social insects. *Evolution*. 65, 3006–3012.

Forsgren, E., Karlsson, A. & Kvarnemo, C. (1996). Female sand gobies gain direct benefits by choosing males with eggs in their nests. *Behavioural Ecology & Sociobiology*. 39, 91–96.

Forster, J. & Hirst, A. G. (2012). The temperature-size rule emerges from ontogenetic differences between growth and development rates. *Functional Ecology*. 26, 483–492.

Forster, J., Hirst, A. G. & Atkinson, D. (2011a). How do organisms change size with changing temperature? The importance of reproductive method and ontogenic timing. *Functional Ecology*. 25, 1024–1031.

Forster, J., Hirst, A. G. & Woodward, G. (2011b). Growth and development rates have different thermal responses. *The American Naturalist*. 178, 668–678.

Foster, G. G. & Suzuki, D. T. (1970). Temperature-sensitive mutations in *Drosophila melanogaster*, IV. A mutation affecting eye facet arrangement in a polarized manner. *Proceedings of the National Academy of Sciences USA*. 67, 738–745.

Fox, C. W. (1994). The influence of egg size on offspring performance in the seed beetle, *Callosobruchus maculatus*. *Oikos*. 71, 321–325.

Fox, C. W., Hickman, D. L., Raleigh, E. L. & Mousseau, T. A. (1995). Paternal investment in a seed beetle (Coleoptera: Bruchidae): influence of male size, age, and mating history. *Annals of the Entomological Society of America*. 88, 100–103.

Fox, C. W., Thakar, M. S. & Mousseau, T. A. (1997). Egg size plasticity in a seed beetle: an adaptive maternal effect. *American Naturalist*. 149, 149–163.

Fox, C. W., Stillwell, R. C., Wallin, W. G. & Hitchcock, L. J. (2006). Temperature and host species affect nuptial gift size in a seed-feeding beetle. *Functional Ecology*. 20, 1003–1011.

Franceskin, V. (2005). *Reconocimiento del sexo y análisis cromosómico en estadios empranos del desarrollo de tefrítidos plaga argentinos*. Masters Dissertation. Facultad de Agronomía, University of Buenos Aires.

Freckleton, R. P., Harvey, P. H. & Pagel, M. (2003). Bergmann's rule and body size in mammals. *The American Naturalist.* 161, 821–825.

French, V., Feast, M. & Partridge, L. (1998). Body size and cell size in *Drosophila:* the developmental response to temperature. *Journal of Insect Physiology.* 44, 1081–1089.

Froman, D. P., Pizzari, T., Feltmann, A. J., Castillo-Juarez, H. & Birkhead, T. R. (2002). Sperm mobility: mechanisms of fertilizing efficiency, genetic variation and phenotypic relationship with male status in the domestic fowl, *Gallus gallus domesticus*. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 269, 607–612.

Gage, M. J. G. (1991). Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. *Animal Behaviour*. 42, 1036–1037.

Gage, M. J. G. (1994). Associations between body size, mating pattern, testis size and sperm lengths across butterflies. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 258, 247–254.

Gage, M. J. G. (1995). Continuous variation in reproductive strategy as an adaptive response to population density in the moth *Plodia interpunctella. Proceedings of the Royal Society of London Series B: Biological Sciences.* 261, 25–30.

Gage, M. J. G. (2012). Complex sperm evolution. *Proceedings of the National Academy of Sciences USA*. 109, 4341–4342.

Gage, M. J. G. & Cook, P. A. (1994). Sperm size or numbers? Effects of nutritional stress upon eupyrene and apyrene sperm production strategies in moth *Plodia interpunctella* (Lepidoptera: Pyralidae). *Functional Ecology.* 8, 594–599.

Gage, M. J. G & Freckleton, R. P. (2003). Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 270, 625–632.

Gage, M. J. G. & Morrow, E. H. (2003). Experimental evidence for the evolution of numerous, tiny sperm via sperm competition. *Current Biology.* 13, 754–757.

Gage, M. J. G., Macfarlane, C. P., Yeates, S., Ward, R. G., Searle, J. B. & Parker, G. A. (2004). Spermatozoal traits and sperm competition in Atlantic Salmon: relative sperm velocity is the primary determinant of fertilization success. *Current Biology.* 14, 44–47.

García-González, F. & Gomendio, M. (2004). Adjustment of copula duration and ejaculate size according to the risk of sperm competition in the golden egg bug (*Phyllomorpha laciniata*). *Behavioural Ecology.* 15, 23–30.

García-González, F. & Simmons, L. W. (2007). Shorter sperm confer higher competitive fertilization success. *Evolution.* 61, 816–824.

Garen, A., Kauvar, L. & Lepesant, J. (1977). Roles of ecdysone in *Drosophila* development. *Proceedings of the National Academy of Sciences USA.* 74, 5099–5103.

Gasparini, C., Simmons, L. W., Beveridge, M. & Evans, J. P. (2010). Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri*. *PLoS ONE*. 5, 1–5.

Gassner, G., Breland, O. P. & Biesele, J. J. (1972). The spermatozoa of the scorpionfly *Panorpa nuptialis*: a transmission electron microscope study. *Annals of the Entomological Society of America*. 65, 1302–1309.

Gavrilets, S., Arnqvist, G. & Friberg, U. (2001). The evolution of female mate choice by sexual conflict. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 268, 531–539.

Gay, L., Hosken, D. J., Vasudev, R., Tregenza, T. & Eady, P. E. (2009). Sperm competition and maternal effects differentially influence testis and sperm size in *Callosobruchus maculatus*. *Journal of Evolutionary Biology*. 22, 1143–1150.

Gay, L., Hosken, D. J., Eady, P. E., Vasudev, R. & Tregenza, T. (2011). The evolution of harm-effect of sexual conflicts and population size. *Evolution*. 65, 725–737.

Gering, R. L. (1953). Structure and function of the genitalia in some American agelenid spiders. *Smithsonian Miscellaneous Collections*. 121, 1–84.

Ghalambor, C. K., McKay, J. K., Carroll, S. P. & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*. 21, 394–407.

Ghiselin, M. T. (2010). The distinction between primary and secondary sexual character. In *The Evolution of Primary Sexual Characters in*

Animals (Eds. Leonard, J. L. & Cordoba-Aguilar, A.) pp. 9–14. Oxford University Press Inc, New York, USA.

Gibert, P., Moreteau, B. & David, J. R. (2000). Developmental constraints on an adaptive plasticity: reaction norms of pigmentation in adult segments of *Drosophila melanogaster*. *Evolution & Development.* 2, 249–260.

Gibert, P., Huey, R. B. & Gilchrist, G. W. (2001). Locomotor performance of *Drosophila melanogaster*: interactions among developmental and adult temperatures, age, and geography. *Evolution.* 55, 205–209.

Giga, D. P. & Smith, R. H. (1987). Egg production and development of *Callosobruchus rhodesianus* (Pic.) and *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) on several commodities at two different temperatures. *Journal of Stored Product Research.* 23, 9–15.

Gilbert, S. F. (2000). *Developmental Biology*. 6th ed. Sinauer, Sunderland, MA.

Gilbert, S. F. (2001). Ecological developmental biology: developmental biology meets the real world. *Developmental Biology*. 233, 1–12.

Gilbert, S. F. (2012). Ecological developmental biology: environmental signals for normal animal development. *Evolution & Development*. 14, 20–28.

Gilchrist, G. W., Huey, R. B. & Partridge, L. (1997). Thermal sensitivity of *Drosophila melanogaster*: evolutionary responses of adults and eggs to laboratory natural selection at different temperatures. *Physiological Zoology*. 70, 403–414.

Gill, J., Kanwar, K. C. & Bawa, S. R. (1971). Abnormal ‘sterile’ strain in *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Annals of the Entomological Society of America.* 64, 1186–1188.

Gomendio, M. & Roldan, E. R. S. (1991). Sperm competition influences sperm size in mammals. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 243, 181–185.

Gomendio, M. & Roldan, E. R. (1993). Coevolution between male ejaculates and female reproductive biology in eutherian mammals. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 252, 7–12.

Gotthard, K. & Nylin, S. (1995). Adaptive plasticity and plasticity as an adaptation: a selective review of plasticity in animal morphology and life history. *Oikos*. 74, 3–17.

Gould, S. J. & Vrba, E. S. (1982). Exaptation-a missing term in the science of form. *Paleobiology*. 8, 4–15.

Grandi, G. (1994). Ovary and testis development in different stages and in the sterile caste of *Kalotermes flavicollis* (Fabr.) (Isoptera: Kalotermitidae). *Ethology Ecology & Evolution, (Special Issue).* 3, 17–23.

Grant, P. R., Grant, B. R. (2007). *How and Why species multiply: The Radiation of Darwin’s Finches Princeton*. New Jersey: Princeton University Press.

Graves, G. R. (1991). Bergmann’s rule near the equator: Latitudinal clines in body size of an Andean passerine bird. *Proceedings of the National Academy of Sciences USA.* 88, 2322–2325.

Grazer, V. M. & Martin, O. Y. (2012a). Investigating Climate Change and Reproduction: Experimental Tools from Evolutionary Biology. *Biology*. 1, 411–438.

Grazer, V. M. & Martin, O. Y. (2012b). Elevated temperature changes female costs and benefits of reproduction. *Evolutionary Ecology*. 26, 625–637.

Green, A. J. (2001). Mass/length residuals: measures of body condition or generators of spurious results? *Ecology*. 82, 1473–1483.

Griffith-Simon, C., and Sheldon, B. C. 2001. Phenotypic plasticity in the expression of sexually selected traits: neglected components of variation. *Animal Behaviour*. 6, 987–993.

Guidi, A. & Rebecchi, L. (1996). Spermatozoan morphology as a character for tardigrade systematics: comparison with sclerified parts of animals and eggs in eutardigrades. *Zoological Journal of the Linnean Society*. 116, 101–113.

Gupta, B. B. & Thapliyal, J. P. (1985). Role of thyroid and testicular hormones in the oxidative metabolism of the Indian garden lizard, *Calotes versicolor*. *General & Comparative Endocrinology*. 58, 20–27.

Hammond, K. A., Szewczak, J. & Krol, E. (2001). Effects of altitude and temperature on organ phenotypic plasticity along an altitudinal gradient. *Journal of Experimental Biology*. 204, 1991–2000.

Harris, W. E., Moore, A. J. & Moore, P. J. (2007). Variation in sperm size within and between ejaculates in a cockroach. *Functional Ecology*. 21, 598–602.

Hebets, E. A. & Papaj, D. R. (2005). Complex signal function: developing a framework of testable hypotheses. *Behavioural Ecology & Sociobiology*. 57, 197–214.

Heino, M. & Kaitala, V. (1999). Evolution of resource allocation between growth and reproduction in animals with intermediate growth. *Journal of Evolutionary Biology.* 12, 423–429.

Helfenstein, F., Szép, T., Nagy, Z., Kempenaers, B. & Wagner, R. H. (2008). Between-male variation in sperm size, velocity and longevity in sand martins Riparia riparia. *Journal of Avian Biology*. 39, 647–652.

Hellriegel, B. & Blanckenhorn, W. U. (2002). Environmental influences on the gametic investment of yellow dung fly males. *Evolutionary Ecology.* 16, 505–522.

Holland, B. & Rice, W. R. (1998). Perspective: chase-away sexual selection: antagonism versus resistance. *Evolution.* 52, 1–7.

Horton, D. R., Lewis, T. M. & Hinojosa, T. (2002). Copulation duration in three species of anthocoris (Heteroptera: Anthocoridae) at different temperatures and effects on insemination and ovarian development. *The Pan-Pacific Entomologist.* 78, 43–55.

Hosken, D. J. & Stockley, P. (2004). Sexual selection and genital evolution. *Trends in Ecology & Evolution.* 19, 87–93.

Hosken, D. J. & Ward, P. I. (2008). Experimental evidence for testis size evolution via sperm competition. *Ecology Letters*. 4, 10–13.

Hosken, D. J., Garner, T. W. J. & Ward, P. I. (2001). Sexual conflict selects for male and female reproductive characters. *Current Biology.* 11, 489–493.

Hosken, D. J., Taylor, M. L., Hoyle, K., Higgins, S. & Wedell, N. (2008). Attractive males have greater success in sperm competition. *Current Biology*. 18. R553–R554.

House, C. M. & Simmons, L. W. (2002). Genital morphology and fertilization success in the dung beetle *Onthophagus taurus*: an example of sexually selected male genitalia. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 270, 447–455.

Hribar, L. J. (1996). Larval rearing temperature affects morphology of *Anopheles albimanus* (Diptera: Culicidae) male genitalia. *Journal of the American Mosquito Control Association.* 12, 295–297.

Huey, R. B., Berrigan, D., Gilchrist, G. W. & Herron, J. C. (1999). Testing the adaptive significance of acclimation: A strong inference approach. *American Zoologist*. 39, 323–336.

Husby, A., Nussey, D. H., Visser, M. E., Wilson, A. J., Sheldon, B. C. & Kruuk, L. E. B. (2010). Contrasting patterns of phenotypic plasticity in reproductive traits in two Great Tit (*Parus major*) populations. *Evolution.* 64, 2221–2237.

Immler, S. & Birkhead, T. R. (2007). Sperm competition and sperm midpiece size: no consistent pattern in passerine birds. *Proceedings of the Royal Society B: Biological Sciences*. 274, 561–568.

Immler, S., Pryke, S. R., Birkhead, T. R. & Griffith, S. C. (2010). Pronounced within-individual plasticity in sperm morphometry across social environments. *Evolution*. 64, 1634–1643.

Immler, S., Gonzalez-Voyer, A. & Birkhead, T. R. (2012). Distinct evolutionary patterns of morphometric sperm traits in passerine

birds. *Proceedings of the Royal Society B: Biological Sciences*. 279, 4174–4182.

Janzen, F. J. (1995). Experimental evidence for the evolutionary significance of temperature dependent sex determination. *Evolution.* 49, 864–873.

Jiao, X. G., Wu, J., Chen, Z. Q., Chen, J. & Liu, F. X. (2009). Effects of temperature on courtship and copulatory behaviours of a wolfspider *Pardosa astrigera* (Araneae: Lycosidae). *Journal of Thermal Biology*. 34, 348–352.

Jones, A. G., Adams, E. M. & Arnold, S. J. (2002). Topping off: A mechanism of first-male sperm precedence in a vertebrate. *Proceedings of the National Academy of Sciences USA.* 99, 2078–2081.

Jordan, M. A. & Snell, H. (2002). Life history trade-offs and phenotypic plasticity in the reproduction of Galapagos lava lizards (*Microlophus delanonis*). *Oecologia.* 130, 44–52.

Karr, T. L. (1991). Intracellular sperm/egg interactions in *Drosophila*: A three-dimensional structural analysis of a paternal product in the developing egg. *Mechanisms of Development*. 34, 101–111.

Katsuki, M. & Miyatake, T. (2009). Effects of temperature on mating duration, sperm transfer and remating frequency in *Callosobruchus chinensis*. *Journal of Insect Physiology.* 55, 112–119.

Kindleman, P., Dixon, A. F. G. & Dostálková, I. (2001). Role of ageing and temperature in shaping reaction norms and fecundity functions in insects. *Journal of Evolutionary Biology.* 14, 835–840.

Kingsolver, J. G. & Huey, R. B. (2008). Size, temperature, and fitness: three rules. *Evolutionary Ecology Research*. 10, 251.

Kingsolver, J. G., Ragland, G. J. & Shlichta, G. J. (2004a). Quantitative genetics of continuous reaction norms: Thermal sensitivity of caterpillar growth rates. *Evolution.* 58, 1521–1529.

Kingsolver, J. G., Izem, R. & Ragland, G. J. (2004b). Plasticity of size and growth in fluctuating thermal environments: Comparing reaction norms and performance curves. *Integrative & Comparative Biology.* 44, 450–460.

Kitamoto, T. (2001). Conditional modification of Behaviour in *Drosophila* by targeted expression of a temperature-sensitive *shibire* allele in defined neurons. *Journal of Neurobiology*. 47, 81–92.

Kleven, O., Laskemoen, T., Fossøy, F., Robertson, R. J. & Lifjeld, J. T. (2008). Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. *Evolution.* 62, 494–499.

Kobayashi, T. (1998). Seasonal changes in body size and male genital structures of *Procladius choreus*. (Diptera: Chironomidae: Tanypodinae). *Aquatic Insects.* 20, 165–172.

Kodric-Brown, A. & Brown, J. H. (1984). Truth in advertising: the kinds of traits favoured by sexual selection. *The American Naturalist.* 124, 309–323.

Kurokawa, H., Matsuo, Y. & Hihara, F. (1974). A study on sperm length and body size of *Drosophila bifasciata*. *Annotationes Zoologicae Japonenses.* 47, 140–146.

Kvist, J., Wheat, C. W., Kallioniemi, E., Saastamoinen, M., Hanski, I. & Frilander, M. J. (2013). Temperature treatments during larval development reveal extensive heritable and plastic variation in gene expression and life history traits. *Molecular Ecology*. 22, 602–619.

LaMunyon, C. W. & Ward, P. I. (1998). Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 265, 1997–2002.

Lance, V. A. & Bogart, M. H. (1992). Disruption of ovarian development in alligator embryos treated with an aromatase inhibitor. *General and Comparative Endocrinology*. 86, 59–71.

Lang, J. W. & Andrews, H. V. (1994). Temperature-dependent sex determination in crocodilians. *Journal of Experimental Zoology.* 270, 28–44.

Lefebvre, L., Juretic, N., Nicolakakis, N. & Timmermans, S. (2001). Is the link between forebrain size and feeding innovations caused by confounding variables? A study of Australian and North American birds. *Animal Cognition.* 4, 91–97.

Leonard, J. L. & Córdoba-Aguilar, A. (2010) *The evolution of primary sexual characters in animals*. Oxford University Press, Oxford, UK.

Levins, R. (1968). *Evolution in changing environments: some theoretical explorations.* Princeton University Press, Princeton, NJ.

Liefting, M., Weerenbeck, M., Van Dooremalen, C. & Ellers, J. (2010). Temperature-induced plasticity in egg size and resistance of eggs to temperature stress in a soil arthropod. *Functional Ecology*. 24, 1291–1298.

Lin, L., Li, H., An, H. & Ji, X. (2008). Do temperature fluctuations during incubation always play an important role in shaping the phenotype of hatchling reptiles? *Journal of Thermal Biology.* 33, 193–199.

Lindsey, C. C. (1966). Body sizes of poikilotherm vertebrates at different latitudes. *Evolution.* 20, 456–465.

Marchinko, K. B. (2003). Dramatic phenotypic plasticity in the barnacle legs (*Balanus glandula Darwin*): magnitude, age dependence, and speed of response. *Evolution.* 57, 1281–1290.

Marchinko, K. B. & Palmer, A. R. (2003). Feeding in flow extremes: dependence of cirrus form on wave-exposure in four barnacle species. *Zoology*. 106, 127–141.

Martel, V., Darrouzet, É. & Boivin, G. (2011). Phenotypic plasticity in the reproductive traits of a parasitoid. *Journal of Insect Physiology*. 57, 682–687.

Martin, O. Y. & Hosken, D. J. (2002). Strategic ejaculation in the common dung fly *Sepsis cynipsea*. *Animal Behaviour.* 63, 541–546.

Mayr, E. (1956). Geographical character gradients and climatic adaptation. *Evolution.* 10, 105–108.

McDonald, A. K. & Nijhout, H. F. (2000). The effect of environmental conditions on mating activity of the Buckeye butterfly, *Precis coenia*. *The Journal of Research on the Lepidoptera.* 35, 22–28.

Meiri, S. & Dayan, T. (2003). On the validity of Bergmann's rule. *Journal of Biogeography.* 30, 331–351.

Meiri, S., Yom-Tov, Y. & Geffen, E. (2007). What determines conformity to Bergmann's rule? *Global Ecology & Biogeography.* 16, 788–794.

Mettke-Hofmann, C., Winkler, H. & Leisler, B. (2002). The significance of ecological factors for explorations and neophobia in parrots. *Ethology*. 108, 240–272.

Michiels, N. K. (1998). Mating conflicts and sperm competition in simultaneous hermaphrodites. In *Sperm competition and sexual selection* (Eds. Birkhead, T. R. & Møller, A. P.) pp. 219–254. Academic Press, San Diego, CA.

Miller, G. T. & Pitnick, S. (2002). Sperm-female coevolution in *Drosophila*. *Science.* 298, 1230–1233.

Minder, A. M., Hosken, D. J. & Ward, P. I. (2005). Co-evolution of male and female reproductive characters across Scathophagidae (Diptera). *Journal of Evolutionary Biology*. 18, 60–69.

Miner, B. G., Sultan, S. E., Morgan, S. G., Padilla, D. K. & Relyea. R. A. (2005). Ecological Consequences of phenotypic plasticity. *Trends in Ecology & Evolution.* 20, 685–692.

Minoretti, N., Stoll, P. & Baur, B. (2013). Heritability of sperm length and adult shell size in the land snail *Arianta arbustorum* (Linnaeus, 1758). *Journal of Molluscan Studies*. Advance online publication. doi:10.1093/mollus/eyt012

Mitchell, N. J., Nelson, N. J., Cree, A., Pledger, S., Keall, S. N. & Daugherty, C. H. (2006). Support for a rare pattern of temperature-dependent sex determination in archaic reptiles: evidence from two species of tuatara (Sphenodon). *Frontiers in Zoology.* 3, 1–12.

Moczek, A. P. (2007). Developmental capacitance, genetic accommodation, and adaptive evolution. *Evolution & Development*. 9, 299–305.

Moczek, A. P. (2008). On the origins of novelty in development and evolution. *BioEssays.* 30, 432–447.

Moczek, A. P. (2010). Phenotypic plasticity and diversity in insects. *Philosophical Transactions of the Royal Society of London*, 365, 593–603.

Moczek, A. P., Sultan, S., Foster, S., Ledón-Rettig, C., Dworkin, I., Nijhout, H. F., Abouheif, E. & Pfennig, D. W (2011). The role of developmental plasticity in evolutionary innovation. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 278, 2705–2713.

Møller, A. P. (1988). Ejaculate quality, testes size and sperm competition in primates. *Journal of Human Evolution.* 17, 479–488.

Møller, A. P. (1989). Ejaculate quality, testes size and sperm production in mammals. *Functional Ecology*. 3, 91–96.

Morrow, E. H. & Gage, M. J. G. (2000). The evolution of sperm lengths in moths. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 267, 307–313.

Morrow, E. H. & Gage, M. J. G. (2001a). Artificial selection and heritability of sperm length in *Gryllus bimaculatus. Heredity.* 87, 356–362.

Morrow, E. H. & Gage, M. J. G. (2001b). Sperm competition experiments between lines of crickets producing different sperm lengths. *Proceedings of the Royal Society of London B.* 268, 2281–2286.

Morrow, E. H. & Gage, M. J. G. (2001c). Consistent significant variation between individual males in spermatozoal morphometry. *Journal of Zoology*. 254, 147–153.

Morrow, E. H., Leijon, A. & Meerupati, A. (2008). Hemiclonal analysis reveals significant genetic, environmental and genotype x environment effects on sperm size in *Drosophila melanogaster*. *Journal of Evolutionary Biology*. 21, 1692–1702.

Muschick, N., Barluenga, M., Salzburger, W. & Meyer, A. (2011). Adaptive phenotypic plasticity in the Midas cichlid fish pharyngeal jaw and its relevance in adaptive radiation. *BMC Evolutionary Biology*. 11, 1–12.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L. & Piferrer, F. (2011). DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLoS Genetics*. 7, 1–15.

Neat, F., Fowler, K., French, V. & Partridge, L. (1995). Thermal evolution of growth efficiency in *Drosophila melanogaster*. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 260, 73–78.

Negovetic, S. & Jokela, J. (2001). Life-history variation, phenotypic plasticity and subpopulation structure in a freshwater snail. *Ecology*. 82, 2805–2815.

Neidhardt, F. C., VanBogelen, R. A. & Vaughn, V. (1984). The genetics and regulation of heat-shock proteins. *Annual Review of Genetics.*18, 295–329.

Neufeld, J. C. & Palmer, R. A. (2008). Precisely proportioned: intertidal barnacles alter penis form to suit coastal wave action. *Proceedings of the Royal Society London B Biological Sciences.* 275, 1081–1087.

Neuwald, J. L. & Valenzuela, N. (2011). The Lesser Known Challenge of Climate Change: Thermal Variance and Sex-Reversal in Vertebrates with Temperature- Dependent Sex Determination. *PLoS ONE*. 6, 1–9.

Nevo, E. (2011). Evolution Under Environmental Stress at Macro-and Microscales. *Genome Biology & Evolution*. 3, 1039–1052.

Niehaus, A. C., Angilletta, M. J., Sears, M. W., Franklin, C. E. & Wilson, R. S. (2012). Predicting the physiological performance of ectotherms in fluctuating thermal environments. *The Journal of Experimental Biology*. 215, 694–701.

Nijhout, H. F. (1999). When developmental pathways diverge. *Proceedings of the National Academy of Sciences USA*. 96, 5348–5350.

Nijhout, H. F. (2003a). Development and evolution of adaptive polyphenisms. *Evolution & Development.* 5, 9–18.

Nijhout, H. F. (2003b). The control of body size in insects. *Developmental Biology.* 261, 1–9.

Nijhout, H. F. & Emlen, D. J. (1998). Competition among body parts in the development and evolution of insect morphology. *Proceedings of the National Academy of Sciences USA*. 95, 3685–3689.

Nijhout, H. F., Roff, D. A. & Davidowitz, G. (2010). Conflicting processes in the evolution of body size and development time. *Philosophical Transactions of the Royal Society of London*. 365, 567–575.

Noach, E. J., de Jong, G. & Scharloo, W. (1997). Phenotypic plasticity of wings in selection lines of *Drosophila melanogaster*. *Heredity.* 79, 1–9.

Nylin, S. & Gotthard, K. (1998). Plasticity in life-history traits. *Annual Reviews Entomology.* 43, 63–83.

Olsson, M., Madsen, T. & Shine, R. (1997). Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 264, 455–459.

Ono, T., Siva-Jothy, M. T. & Kato, A. (1989). Removal and subsequent ingestion of rivals' semen during copulation in a tree cricket. *Physiological Entomology.* 14, 195–202.

Oostra, V., de Jong, M. A., Invergo, B. M., Kesbeke, F., Wende, F., Brakefield, P. M. & Zwaan, B. J. (2011). Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly. *Proceedings of the Royal Society B: Biological Sciences*. 278, 789–797.

Oppliger, A., Hosken, D. J. & Ribi, G. (1998). Snail sperm production characteristics vary with sperm competition risk. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 265, 1527–1534.

Oppliger, A., Naciri-Graven, Y., Ribi, G. & Hosken, D. J. (2003). Sperm length influences fertilization success during sperm competition in the snail *Viviparus ater*. *Molecular Ecology*. 12, 485–492.

Otronen, M. (1998). Male asymmetry and postcopulatory sexual selection in the fly *Dryomyza anilis*. *Behavioural Ecology & Sociobiology*. 42, 185–191.

Parker, G. A. (1970a). Sperm competition and its evolutionary consequences in insects. *Biological Reviews.* 45, 525–567.

Parker, G. A. (1970b). The reproductive behaviour and the nature of sexual selection in *Scatophaga stercoraria* L. (Diptera: Scatophagidae). VII. The origin and evolution of the passive phase. *Evolution*. 24, 774–788.

Parker, G. A. (1970c). Sperm competition and its evolutionary effect on copula duration in the fly *Scatophaga stercoraria*. *Journal of Insect Physiology*. 16, 1301–1328.

Parker, G. A. (1971). The reproductive behaviour and the nature of sexual selection in *Scatophaga stercoraria* L. (Diptera: Scatophagidae): *The Journal of Animal Ecology*. 40, 215–223.

Parker, G. A. (1979). Sexual selection and sexual conflict. In *Sexual selection and reproductive competition in insects* (Eds. Blum, M. S. & Blum, N. S.) pp. 123–166. Academic, New York.

Parker, G. A. (1982). Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. *Journal of Theoretical Biology*. 96, 281–294.

Parker, G. A. (1992). Marginal value theorem with exploitation time costs: diet, sperm reserves, and optimal copula duration in dung flies. *The American Naturalist*. 139, 1237–1256.

Parker, G. A. (2006). Sexual conflict over mating and fertilization: an overview. *Philosophical Transactions of the Royal Society of London B.* 361, 235–259.

Parker, G. A. & Simmons, L. W. (1991). A model of constant random sperm displacement during mating: evidence from Scatophaga. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 246, 107–115.

Parker, G. A. & Simmons, L. W. (1994). Evolution of phenotypic optima and copula duration in dungflies. *Nature*. 370, 53–56.

Parker, J. & Johnston, L. A. (2006). The proximate determinants of insect size. *Journal of Biology*. 5, 1–4.

Parker, G. A., Simmons, L. W. & Kirk, H. (1990). Analysing sperm competition data: simple models for predicting mechanisms. *Behavioural Ecology & Sociobiology*. 27, 55–65.

Parker, G. A., Simmons, L. W., Stockley, P., McChristie, D. M. & Charnov, E. L. (1999). Optimal copula duration in yellow dung flies: effects of female size and egg content. *Animal Behaviour*. 57, 795–805.

Partridge, L., Barrie, B., Fowler, K. & French, V. (1994). Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. *Evolution*. 48, 1269–1276.

Partridge, L. & French, V. (1996). Thermal evolution of ectotherm body size: Why get big in the cold? In *Animals and temperature: Phenotypic and evolutionary adaptation* (Eds. Johnston, I. A. & Bennett, A. F.) pp. 265–292. Cambridge University Press, Cambridge.

Pattarini, J. M., Starmer, W. T., Bjork, A. & Pitnick, S. (2006). Mechanisms underlying the sperm quality advantage in *Drosophila melanogaster*. *Evolution*. 60, 2064–2080.

Peacor, S. D. & Werner, E. E. (2003). The contribution of trait-mediated indirect effects to the net effects of a predator. *Proceedings of the National Academy of Sciences USA*. 98, 3904–3908.

Penttilä, A., Slade, E. M., Simojoki, A., Riutta, T., Minkkinen, K. & Roslin, T. (2013). Quantifying Beetle-Mediated Effects on Gas Fluxes from Dung Pats. *PLoS ONE*. 8, e71454.

Perez-Velazquez, M., Bray, W. A., Lawrence, A. L., Gatlin III, D. M., & Gonzalez-Felix, M. L. (2001). Effect of temperature on sperm quality of captive *Litopenaeus vannamei* broodstock. *Aquaculture*. 198, 209-218.

Perje, A. M. (1948). Studies on the spermatogenesis in *Musca domestica. Hereditas.* 34, 209–232.

Pétavy, G., David, J. R., Debat, V., Gibert, P. & Moreteau, B. (2004). Specific effects of cycling stressful temperatures upon phenotypic and genetic variability of size traits in *Drosophila melanogaster. Evolutionary Ecology Research.* 6, 1–8.

Petrie, M. (1994). Improved growth and survival of offspring of peacocks with more elaborate trains. *Nature*. 371, 598–599.

Pfennig, D. W., Wund, M. A., Snell-Rood, E. C., Cruickshank, T., Schlichting, C. D. & Moczek, A. P. (2010). Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology & Evolution.* 25, 459–467.

Pieau, C. (1996). Temperature variation and sex determination in reptiles. *Bioessays.* 18, 19–26.

Pieau, C. & Dorizzi, M. (1981). Determination of temperature sensitive phases for sexual differentiation of the gonads in the embryo of the turtle, *Emys orbicularis. Journal of Morphology.* 170, 373–382.

Pieau, C., & Dorizzi, M. (2004). Oestrogens and temperature-dependent sex determination in reptiles: all is in the gonads. *Journal of Endocrinology*. 181, 367–377.

Pieau, C., Dorizzi, M. & Richard-Mercier, N. (1999). Temperature-dependent sex determination and gonadal differentiation in reptiles. *Cellular & Molecular Life Sciences.* 55, 887–900.

Pieau, C., Dorizzi, M., & Richard-Mercier, N. (2001). Temperature-dependent sex determination and gonadal differentiation in reptiles. In *Genes and Mechanisms in Vertebrate Sex Determination* (Eds Scherer, G. & Schmid, G.) pp. 117–141. Basel: Bikhäuser Verlag .

Pigliucci, M. (1996). How organisms respond to environmental changes: from phenotypes to molecules (and vice versa). *Trends in Ecology & Evolution.* 11, 168–173.

Pigliucci, M. (2001). *Phenotypic plasticity: beyond nature and nurture.* Baltimore, MD: Johns Hopkins University Press.

Pigliucci, M. (2005). Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology & Evolution*. 20, 481–486.

Pincheira-Donoso, D. (2010). The balance between predictions and evidence and the search for universal macroecological patterns: taking Bergmann's rule back to its endothermic origin. *Theory in Biosciences.* 129, 247–253.

Pincheira-Donoso, D., Tregenza, T. & Hodgson, D. J. (2007). Body size evolution in South American *Liolaemus* lizards of the *boulengeri* clade: a contrasting reassessment. *Journal of Evolutionary Biology.* 20, 2067–2071.

Pincheira-Donoso, D., Hodgson, D. J. & Tregenza, T. (2008). The evolution of body size under environmental gradients in ectotherms: why should Bergmann's rule apply to lizards? *BMC Evolutionary Biology*. 8, 1–13.

Pitnick, S. & Markow, T. A. (1994). Male gametic strategies: sperm size, testes size, and the allocation of ejaculate among successive mates by the sperm-limited fly *Drosophila pachea* and its relatives. *American Naturalist*. 143, 785–819.

Pitnick, S., Spicer, G. S. & Markow, T. A. (1995). How long is a giant sperm? *Nature.* 375, 109.

Pitnick, S., Markow, T. A. & Spicer, G. S. (1999). Evolution of multiple kinds of female sperm-storage organs in *Drosophila. Evolution.* 53, 1804–1822.

Pitnick, S., Miller, G. T., Reagan, J. & Holland, B. (2001). Males' evolutionary responses to experimental removal of sexual selection. *Proceedings of the Royal Society of London Series B: Biological Sciences*. 268, 1071–1080.

Pitnick, S., Hosken, D. J. & Birkhead, T. R. (2009). Sperm morphological diversity. In *Sperm Biology: An Evolutionary Perspective* (Eds. Birkhead, T. R., Hosken D. J. & Pitnick, S.) pp. 69–149. Academic Press, London.

Pizzari, T. & Birkhead, T. R. (2000). Female feral fowl eject sperm of subdominant males. *Nature*. 405, 787–789.

Powers, D. A., & Schulte, P. M. (1998). Evolutionary adaptations of gene structure and expression in natural populations in relation to a changing environment: a multidisciplinary approach to address the million-year saga of a small fish. *The Journal of Experimental Zoology*. 282, 71–94.

Presgraves, D. C., Baker, R. H. & Wilkinson, G. S. (1999). Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies.

Proceedings of the Royal Society of London Series B: Biological Sciences. 266, 1041–1047.

Price, T. D., Qvarnström, A & Irwin, D. E. (2003). The role of phenotypic plasticity in driving genetic evolution. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 270, 1433–1440.

Purchase, C. F., Butts, I. A., Alonso-Fernandez, A. & Trippel, E. A. (2010). Thermal reaction norms in sperm performance of Atlantic cod (Gadus morhua).*Canadian Journal of Fisheries & Aquatic Sciences*. 67, 498–510.

Radwan, J. (1996). Intraspecific variation in sperm competition success in the bulb mite: a role for sperm size. *Proceedings of the Royal Society of London Series B: Biological Sciences.* 263, 855–859.

Ramm, S. A. & Stockley, P. (2009). Adaptive plasticity of mammalian sperm production in response to social experience. *Proceedings of the Royal Society B: Biological Sciences*. 276, 745–751.

Ransford, M. O. (1997). *Sperm Competition in the 2-spot Ladybird, Adalia bipunctata* (Doctoral dissertation, University of Cambridge).

Reiskind, M. H. & Zarrabi, A. A. (2012). Is bigger really bigger? Differential responses to temperature in measures of body size of the mosquito, *Aedes albopictus. Journal of Insect Physiology.* 58, 911–917.

Relyea, R. A. (2002). Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. *Ecological Monographs.* 72, 77–93.

Relyea, R. A. (2004). Fine-tuned phenotypes: tadpole plasticity under 16 combinations of predators and competitors. *Ecology*. 85, 172–179.

Relyea, R. & Auld, J. R. (2004). Having the guts to compete: how intestinal plasticity explains costs of inducible defences. *Ecology Letters.* 7, 869–875.

Rice, W. R. (1996). Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*. 381, 232–234.

Rice, W. R., & Holland, B. (1997). The enemies within: intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen. *Behavioural Ecology & Sociobiology*. 41, 1–10.

Ridley, M. (1988). Mating frequency and fecundity in insects. *Biological Reviews.* 63, 509–549.

Robertson, F. W. (1955). The ecological genetics of growth in *Drosophila* 2. Selection for large body size on different diets. *Genetical Research*. 1, 305–318.

Robertson, F. W. (1959). Studies in quantitative inheritance. XII. Cell size and number in relation to genetic and environmental variation of body size in *Drosophila*. *Genetics*. 44, 869–896.

Rodríguez, R. L. (2012). Grain of environment explains variation in the strength of genotype x environment interaction. *Journal of Evolutionary Biology*. 25, 1897–1901.

Roff, D. A. (1980). Optimizing development time in a seasonal environment: the ‘ups and downs’ of clinal variation. *Oecologia.* 45, 202–208.

Roff, D. A. (1986). Predicting body size with life history models. *Bioscience.* 36, 316–323.

Roff, D. A. (2002). *Life History Evolution.* Sinnauer Associates, Inc., Sunderland, Massachusetts.

Roldan, E. R. S., Gomendio, M. & Vitullo, A. D. (1992). The evolution of eutherian spermatozoa and underlying selective forces: female selection and sperm competition. *Biological Reviews*. 67, 551–593.

Rönn, J., Katvala, M. & Arnqvist, G. (2011). Correlated evolution between male and female primary reproductive characters in seed beetles. *Functional Ecology.* 25, 634–640.

Rosenzweig, M. L. (1968). The strategy of body size in mammalian carnivores. *American Midland Naturalist.* 80, 299–315.

Rovner, J. S. (1971). Mechanisms controlling copulatory Behaviour in Wolf Spiders (Araneae: Lycosidae). *Psyche: A Journal of Entomology.* 78, 150–165.

Rudolfsen, G., Figenschou, L., Folstad, I. & Kleven, O. (2008). Sperm velocity influence paternity in the Atlantic cod (*Gadus morhua*) (L.). *Aquaculture Research.* 39, 212–216.

Rugman-Jones, P. F. & Eady, P. E. (2008). Co-evolution of male and female reproductive traits across the Bruchidae (Coleoptera). *Functional Ecology.* 22, 880–886.

Rurangwa, E., Kime, D. E., Ollevier, F. & Nash, J. P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture*. 234, 1–28.

Saeki, Y., Kruse, K. C. & Switzer, P. V. (2005). Physiological costs of mate guarding in the Japanese beetle (*Popillia japonica Newman*). *Ethology*. 111, 863–877.

Savalli, U. M. & Fox, C. W. (1999). The effect of male mating history on paternal investment, fecundity and female remating in the seed beetle *Callosobruchus maculatus*. *Functional Ecology*. 13, 169–177.

Schärer, L., Littlewood, D. T. J., Waeschenbach, A., Yoshida, W. & Vizoso, D. B. (2011). Mating behavior and the evolution of sperm design. *Proceedings of the National Academy of Sciences USA*. 108, 1490–1495.

Scheiner, S. M. (1993). Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology & Systematics*. 24, 35–68.

Schlichting, C. D. & Pigliucci, M. (1995). Gene regulation, quantitative genetics and the evolution of reaction norms. *Evolutionary Ecology*. 9, 154–168.

Schlichting, C. D. & Smith, H. (2002). Phenotypic plasticity: linking molecular mechanisms with evolutionary outcomes. *Evolutionary Ecology*. 16, 189–211.

Schmalhausen, I. I. (1949). *Factors of Evolution*. Blakiston, Philadelphia.

Schütt, C. & Nöthiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. *Development*. 127, 667–677.

Schutze, M. K. & Clarke, A. R. (2008). Converse Bergmann cline in a eucalyptus herbivore, *Paropsis atomaria Olivier* (Coleoptera: Chrysomelidae): phenotypic plasticity or local adaptation? *Global Ecology & Biogeography*. 17, 424–431.

Sevenster, J. G. (1995). Equations or organisms? A comment on Berrigan and Charnov. *Oikos*. 73, 405–407.

Sheader, M. (1996). Factors influencing egg size in the gammarid amphipod *Gammarus insensibilis*. *Marine Biology.* 124, 519–526.

Shine, R. (2004). Seasonal shifts in nest temperature can modify the phenotypes of hatchling lizards, regardless of overall mean incubation temperature. *Functional Ecology.* 18, 43–49.

Shine, R., Olsson, M. M. & Mason, R. T. (2008). Chastity belts in gartersnakes: the functional significance of mating plugs. *Biological Journal of the Linnean Society*. 70, 377–390.

Shoemaker, C. M. & Crews, D. (2009). Analyzing the coordinated gene network underlying temperature-dependent sex determination in reptiles. *Seminars in Cell & Developmental Biology*. 20, 293–303.

Shubin, N., Tabin, C. & Carroll, S. (2009). Deep homology and the origins of evolutionary novelty. *Nature*. 457, 818–823.

Sih, A., Lauer, M. & Krupa, J. (2002). Path analysis and the relative importance of male-female conflict, female choice and male-male competition in water striders. *Animal Behaviour.* 63, 1079–1089.

Simmons, L. W. (2001). *Sperm Competition and Its Evolutionary Consequences in Insects*. Princeton, NJ: Princeton University Press.

Simmons, L. W. & Emlen, D. J. (2006). Evolutionary trade-off between weapons and testes. *Proceedings of the National Academy of Sciences USA.* 103, 16346–16351.

Simmons, L. W. & Kotiaho, J. S. (2002). Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution.* 56, 1622–1631.

Simmons, L.W. & Siva-Jothy, M. T. (1998). Sperm competition in insects: mechanisms and the potential for selection. In *Sperm Competition and Sexual Selection* (Eds. Birkhead, T.R. & Møller, A.P.) pp. 341–434. Academic Press, London.

Skinner, A. M. J. & Watt, P. J. (2007). Phenotypic correlates of spermatozoon quality in the guppy, *Poecilia reticulata. Behavioural Ecology.* 18, 47–52.

Snook, R. R. (2005). Sperm competition: not playing by the numbers. *Trends in Ecology & Evolution.* 20, 46–53.

Snook, R. R., Gidaszewski, N. A., Chapman, T. & Simmons, L. W. (2013). Sexual selection and the evolution of secondary sexual traits: sex comb evolution in *Drosophila*. *Journal of Evolutionary Biology*. 26, 912–918.

Snow, L. S. & Andrade, M. C. (2004). Pattern of sperm transfer in redback spiders: implications for sperm competition and male sacrifice. *Behavioural Ecology*. 15, 785–792.

Somero, G. N. (1995). Proteins and temperature. *Annual Review of Physiology.* 57, 43–68.

Stearns, S. C. (1992). *The evolution of life histories.* Oxford University Press, Oxford.

Stearns, S. C. & Koella, J. C. (1986). The evolution of phenotypic plasticity in life-history traits: predictions of reaction norms for age and size at maturity. *Evolution.* 40, 893–913.

Steigenga, M. J. (2008). *Adaptation or physiological constraint: Temperature-mediated plasticity in reproduction*. PhD Thesis, Universitätsbibliothek, Austria.

Stevens, D. J. (2004). Pupal development temperature alters adult phenotype in speckled wood butterfly, *Pararge aegeria*. *Journal of Thermal Biology*. 29, 205–210.

Stevens, M. E. & Bryant, P. J. (1986). Temperature-dependent expression of the apterous phenotype in *Drosophila melanogaster*. *Genetics*. 112, 217–228.

Stillwell, R. C. & Fox, C. W. (2005). Complex patterns of phenotypic plasticity: Interactive effects of temperature during rearing and oviposition. *Ecology*. 86, 924–934.

Stillwell, R. C. & Fox, C. W. (2007). Environmental effects on sexual size dimorphism of a seed-feeding beetle. *Oecologia*. 153, 273–280.

Stillwell, R. C., Wallin, W. G., Hitchcock, L. J. & Fox, C. W. (2007). Phenotypic plasticity in a complex world: interactive effects of food and temperature on fitness components of a seed beetle. *Oecologia*. 153, 309–321.

Stillwell, R. C., Moya-Laraño, J. & Fox, C. W. (2008). Selection does not favour larger body size at lower temperature in a seed-feeding beetle. *Evolution*. 62, 2534–2544.

Stillwell, R. C., Blanckenhorn, W. U., Teder, T., Davidowitz, G. & Fox, C. W. (2010). Sex differences in phenotypic plasticity affect variation in sexual size dimorphism in insects: from physiology to evolution. *Annual Review of Entomology*. 55, 227–245.

Stockley, P. (1997). Sexual conflict resulting from adaptations to sperm competition. *Trends in Ecology & Evolution*. 12, 154–159.

Stockley, P., Gage, M. J. G., Parker, G. A. & Møller, A. P. (1997). Sperm competition in fishes: The evolution of testis size and ejaculate characteristics. *The American Naturalist.* 149, 933–954.

Stockley, P. & Seal, N. J. (2001). Plasticity in reproductive effort of male dung flies (*Scatophaga stercoraria*) as a response to larval density. *Functional Ecology*. 15, 96–102.

Sultan, S. E. (1995). Phenotypic plasticity and plant adaptation. *Acta Botanica Neerlandica.* 44, 363–383.

Sultan, S. E. (2000). Phenotypic plasticity for plant development, function and life history. *Trends Plant Science.* 5, 537–542.

Sultan, S. E. (2003). Phenotypic plasticity in plants: a case study in ecological development. *Evolution & Development.* 5, 25–33.

Sultan, S. E. & Spencer, H. G. (2002). Metapopulation structure favours plasticity over local adaptation. *The American Naturalist.* 160, 271–283.

Suzuki, Y. & Nijhout, H. F. (2006). Evolution of a polyphenism by genetic accommodation. *Science.* 311, 650–652.

Swanson, W. J. & Vacquier, V. D. (2002). The rapid evolution of reproductive proteins. *Nature Reviews Genetics*. 3, 137–144.

Switzer, P. V., Enstrom, P. C. & Schoenick, C. A. (2008). Environmental conditions affect sperm competition risk in Japanese beetles (Coleoptera: Scarabidae). *Annals of the Entomological Society of America.* 101, 1154–1161.

Takakura, K. (2001). Courtship-role-reversal in the bean weevil, *Bruchidius dorsalis* (Coleoptera: Bruchidae): interplay between male-male

competition and cryptic female choice. *Applied Entomology & Zoology.* 36, 311–316.

Thompson, D. B. (1992). Consumption rates and the evolution of diet-induced plasticity in the head morphology of *Melanoplus femurrubrum* (Orthoptera: Acrididae). *Oecologia.* 89, 204–213.

Thornhill, R. (1983). Cryptic female choice and its implications in the scorpionfly *Hylobittacus nigriceps*. *The American Naturalist*. 122, 765–788.

Thornhill, R. (1984). Alternative hypotheses for traits believed to have evolved by sperm competition. In *Sperm Competition and the Evolution of Animal Mating Systems* (ed. Smith, R. L.), pp. 151–178, Academic Press, Orlando.

Thornhill, R. & Sauer, K. P. (1991). The notal organ of the scorpionfly (*Panorpa vulgaris*): an adaptation to coerce mating duration. *Behavioural Ecology*. 2, 156–164.

Tregenza, T. & Wedell, N. (2000). Genetic compatibility, mate choice and patterns of parentage: invited review. *Molecular Ecology*. 9, 1013–1027.

Trivers, R. L. (1972). Parental investment and sexual selection. In *Sexual Selection and the Descent of Man* (ed. Campbell, B.), pp. 136–179. Chicago: Aldine.

Tsubaki, Y., Samejima, Y. & Siva-Jothy, M. T. (2010). Damselfly females prefer hot males: higher courtship success in males sunspots. *Behavioural Ecology & Sociobiology.* 64, 1547–1554.

Turner, L. M. & Hoekstra, H. E. (2008). Causes and consequences of the evolution of reproductive proteins. *International Journal of Developmental Biology*. 52, 769–780.

Ueno, H. (1994). Intraspecific variation of P2 value in a coccinellid beetle, *Harmonia axyridis*. *Journal of Ethology*. 12, 169–174.

Valenzuela, N. (2001). Constant, shift, and natural temperature effects on sex determination in *Podocnemis expansa* turtles. *Ecology.* 82, 3010–3024.

van der Have, T. M. & de Jong, G. (1996). Adult size in ectotherms: temperature effects on growth and differentiation. *Journal of Theoretical Biology.* 183, 329–340.

Van Dooremalen, C., Koekkoek, J. & Ellers, J. (2011). Temperature-induced plasticity in membrane and storage lipid composition: Thermal reaction norms across five different temperatures. *Journal of Insect Physiology*. 57, 285–291.

Vermeulen, A., Engels, S., Engqvist, L. & Sauer, K. P. (2009). Phenotypic plasticity in sperm traits in scorpionflies (Mecoptera: Panorpidae): consequences of larval history and seasonality on sperm length and sperm transfer. *European Journal of Entomology.* 106, 347–352.

Via, S., Gomulkiewicz, R., de Jong, G., Scheiner, S. M., Schlichting, C. D. & Van Tienderan, P. H. (1995). Adaptive phenotypic plasticity: consensus and controversy. *Trends in Ecology & Evolution.* 10, 212–217.

Voordouw, M. J., Koella, J. C. & Hurd, H. (2008). Intra-specific variation of sperm length in the malaria vector *Anopheles gambiae*: males with shorter sperm have higher reproductive success. *Malaria Journal.* 7, 1–12.

Waage, J. K. (1979). Dual function of the damselfly penis: sperm removal and transfer. *Science.* 203, 916–918.

Waddington, C. H. (1942). Canalization of development and the inheritance of acquired characters. *Nature*. 150, 563–565.

Walters, R. J. & Hassall, M. (2006). The temperature-size rule in ectotherms: May a general explanation exist after all. *The American Naturalist.* 167, 510–523.

Ward, J. V. (1982). Thermal responses in the evolutionary ecology of aquatic insects. *Annual Review of Entomology.* 27, 97–117.

Ward, P. I. & Hauschteck-Jungen, E. (1993). Variation in sperm length in the yellow dung fly *Scathophaga stercoraria* (L.)**.** *Journal of Insect Physiology.* 39, 545–547.

Ward, P. I. & Simmons, L. W. (1991). Copula duration and testes size in the yellow dung fly, *Scathophaga stercoraria* (L.): the effects of diet, body size and mating history. *Behavioural Ecology & Sociobiology.* 29, 77–85.

Warner, D. A. & Shine, R. (2008). The adaptive significance of temperature-dependent sex determination in a reptile. *Nature.* 451, 566–568.

Wedell, N., Gage, M. J. G. & Parker, G. A. (2002). Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution*. 17, 313–320.

West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution.* Oxford University Press, New York.

West-Eberhard, M. J. (2005a). Developmental plasticity and the origin of species differences. *Proceedings of the National Academy of Sciences USA.* 102, 6543–6549.

West-Eberhard, M. J. (2005b). Phenotypic accommodation: adaptive innovation due to developmental plasticity. *Journal of Experimental Zoology Part B: Molecular & Developmental Evolution*. 304, 610–618.

Whitman, D. W. & Agrawal, A. A. (2009). "What Is Phenotypic Plasticity and Why Is It Important?" In *Phenotypic Plasticity of Insects: Mechanisms & Consequences* (Eds. Whitman, D. W. & Ananthakrishnan, T. N.) pp. 1–63. Science Publishers.

Wilder, S. M. & Rypstra, A. L. (2007). Male control of copulation duration in a wolf spider (Araneae, Lycosidae). *Behaviour*. 144, 471–484.

Wilson, K. & Hill, L. (1989). Factors affecting egg maturation in the bean weevil *Callosobruchus maculatus*. *Physiological Entomology.* 14, 115–126.

Wilson, N., Tubman, S. C., Eady, P. E. & Robertson, G. W. (1997). Female genotype affects male success in sperm competition. *Proceedings of the Royal Society B: Biological Sciences*. 264, 1491–1495.

Witalis, J. & Godula, J. (1993). Postembryonal development of the testes in cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Noctuidae: Lepidoptera). *Acta Biologica Hungarica*. 44, 281–295.

Wolfner, M. F. (1997). Tokens of love: functions and regulation of Drosophila male accessory gland products. *Insect Biochemistry & Molecular Biology*. 27, 179–192.

Woods, H. A. (1999). Egg-mass size and cell size: effects of temperature on oxygen distribution. *American Zoologist*. 39, 244–252.

Yamahira, K. & Conover, D. O. (2002). Intra- vs. interspecific latitudinal variation in growth: Adaptation to temperature or seasonality? *Ecology*. 83, 1252–1262.

Yao, H. H., DiNapoli, L. & Capel, B. (2004). Cellular mechanisms of sex determination in the red-eared slider turtle, *Trachemys scripta*. *Mechanisms of Development*. 121, 1393–1401.

Yntema, C. L. & Mrosovsky, N. (1982). Critical periods and pivotal temperatures for sexual differentiation in loggerhead sea turtles. *Canadian Journal of Zoology*. 60, 1012–1016.

Zamudio, K. R., Huey, R. B. & Crill, W. D. (1995). Bigger isn't always better: body size, developmental and parental temperature and male territorial success in *Drosophila melanogaster*. *Animal Behaviour*. 49, 671–677.

Zeh, J. A. & Zeh, D. W. (1996). The evolution of polyandry I: intragenomic conflict and genetic incompatibility. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 263, 1711–1717.

Zeh, J. A. & Zeh, D. W. (1997). The evolution of polyandry II: post–copulatory defenses against genetic incompatibility. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 264, 69–75.

Zera, A. J. & Harshman, L. G. (2001). The physiology of life history trade-offs in animals. *Annual Review of Ecological Systems.* 32, 95–126.

Zera, A. J., Potts, J. & Kobus, K. (1998). The physiology of life-history trade-offs: Experimental analysis of a hormonally induced life-history trade-off in *Gryllus assimilis. The American Naturalist.* 152, 7–23.

Zwaan, B. J., Azevedo, R. B. R., James, A. C., van 't Land, J. & Partridge, L. (2000). Cellular basis of wing size variation in *Drosophila melanogaster*: A comparison of latitudinal clines on two continents. *Heredity*. 84, 338–347.